CXXXVI. STRUCTURE AND ENZYME REACTIONS. PARTS I AND II.

THE SYSTEMS UREA-UREASE-CHARCOAL AND POLYSACCHARIDE-AMYLASE-CHARCOAL.

By STANISLAUS JOHN PRZYLECKI, HEDWIGA NIEDZWIEDZKA and THADDEUS MAJEWSKI.

From the Biochemical Laboratory, Faculty of Veterinary Medicine, Warsaw University.

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The question of the applicability of the law of mass action to reactions in the living organism has long been considered as one of the most important problems in the dynamics of life phenomena.

The chief difficulty in the solution of this problem is that these reactions take place in a heterogeneous medium, in which, apart from purely chemical reactions, we encounter processes of diffusion, and the whole question is further complicated by the fact that most of the reacting substances are in the colloidal state.

For this reason, all except ionic reactions and those connected with the last stages of disintegration or the first stages of synthesis, are reactions involving all those factors connected with reactions of hydrophile colloids in heterogeneous media, *i.e.* reactions of adsorption, and in general, all surface phenomena, to which the law of mass action is, at least in the state of presen day knowledge, inapplicable.

In this paper we shall consider the questions of the applicability of the law of mass action to enzyme reactions. In general, in so far as the kinetics of enzyme reactions in homogeneous systems are concerned, if we can, in general, consider any enzyme system to be homogeneous, these conform to the law of mass action, as has been pointed out by Michaelis and Menten [1913] and Josephson [1925].

It is a question whether the same holds good for enzyme reactions *in vivo*, and whether reversible enzyme reactions may here be interpreted on the basis of the concentration of the substrates. Here we encounter a whole series of incomprehensible facts, apparently irreconcilable with the law of mass action, a classical example of this being the different behaviour of amylase *in vitro* and *in vivo*.

Some factor must therefore exist in the structure of the cell which alters the course of reactions and their final equilibrium point. This factor need not necessarily be connected either with the nervous or the endocrine system, but may be connected with the actual structure of the liver cell. A whole series of examples could be cited, showing that this phenomenon is not limited to amylase, but may also be encountered among the most varied reactions of enzymic hydrolysis, involving colloidal constituents, such as proteins, carbohydrates and nucleic acids.

Apart from the question of the direction of the reaction and its final equilibrium point, it is a point of considerable interest that the velocity of enzyme reactions depends to a large extent upon the structural conditions of the environment in which the enzyme acts. In general, enzymic and vital processes may be classified into those in which the velocity of reaction is accelerated by the destruction of structure, and those in which it is retarded, most hydrolytic ferments belonging to the former class.

Hofmeister [1901], on the basis of Bütschli's theory of the alveolar structure of protoplasm, considers that reactions take place in the more liquid portions of the cell. Thus ferments, to which the colloidal parts of the partitions are impermeable, cannot liberate themselves from the latter, in contrast with those crystalloids, taking part in the reactions of the cell, which are able freely to circulate.

Hofmeister did not analyse more closely the possible causes of the binding of the enzymes. These may be extremely heterogeneous. Hedin [1915] was one of the first to draw attention to the phenomenon of adsorption as a possible factor in the explanation of variability of the velocity of enzyme reactions *in vivo*.

Lesser [1920] showed that the retardation of the decomposition of glycogen may take place, not only *in vivo*, but may also be reproduced experimentally by the addition of some adsorbent to the solution. Lesser interpreted his results in the following way. Only that portion of ferment which is in solution is active, the adsorbed portion being inactive. Substances which lower surface tension increase the active concentration of ferment by elution of the latter. This effect is similar to the hypoglycaemia produced by alcohol and other narcotics *in vivo*.

This phenomenon has been the subject of numerous researches, based upon the assumption that adsorbed enzymes are inactive. Certain facts tending to discredit this assumption may, however, be found, such as Meyerhof's [1914] discovery that saccharase, adsorbed on colloidal ferric hydroxide, retains its enzymic activity. Admitting therefore, that adsorption need not necessarily deprive enzymes of their activity, the interpretation of Lesser's experiments becomes exceedingly difficult.

The possibility may arise that, if enzymes lose their activity not through adsorption, but in accordance with Hofmeister's [1901] conception, at least in certain cases, changes in the velocity of enzyme reaction in heterogeneous

systems may be due to the separation of the ferment from its substrate. In that case, we should have to admit that this retarding effect would arise when both the enzyme and its substrate are adsorbed, and this appears to be the case for the system amylase-glycogen.

Certain unpublished experiments carried out by one of us [cf. Przylecki, 1922], in 1918–1920, also support this interpretation. In these experiments we found that, during the months in which Lesser found almost complete absence of glycogenolytic action in the frog's liver, *i.e.* from November to January, the liver synthesises glycogen quite efficiently from glucose. Thus frogs placed in 7 % glucose at 15° exhibited a considerable increment of glycogen, the content of the latter in the liver increasing on the average by 40 % over a period of two weeks. Almost identical results were obtained in experiments in which the glycogen content of the liver was determined at the beginning of the experiment, using in this case summer frogs at the same temperature (see Table I).

Table I. *Glycogen content of lobes of the livers of frogs kept* 10 *days in a* 7 % *solution of glucose at* 15°.

Month	No. of experiments	Content in the analysed lobe at beginning of experiment in % live weight		Same after ten days	
		Average	Deviations	Average	Deviations
December	5	5·5	4·8–6·0	7·8	7·1–8·3
June–July	5	4·8	3·9–5·9	6·8	5·3–7·9

If therefore, Lesser's hypothesis were correct, it could be reconciled with our findings only on the assumption that the synthesis of glycogen depends upon a different enzyme from amylase, since any other interpretation would necessarily involve accepting the view that a free ferment acts as efficiently as when adsorbed. The difference in the velocity of hydrolysis of glycogen between summer and winter livers would not, in that case, be due to differences in the amount of amylase present, but to the distance separating the enzyme from its substract. Should this be so, it would be clear that the enzyme acts equally efficiently for the synthesis as for the hydrolysis of glycogen, and further, the reason for the existence of seasonal differences in the velocity of the latter process would be explained, since the enzyme and substrate may be more or less separated from one another, according to the season.

In order to decide which of the above views is correct, *i.e.* to prove that the velocity of enzyme reactions is affected, not by adsorption of the enzyme, but by spatial separation of the latter from the substrate, a whole series of experiments would be necessary.

The experimental part of this research has as its object the verification of the following postulates upon which the above reasoning is based.

1. That adsorbed enzymes retain their activity; *i.e.* that the velocity of enzyme reactions, in systems where the enzyme is strongly adsorbed on to

some adsorbent, and the substrate slightly or not at all, is equal to or only slightly less than in the absence of the adsorbent.

2. That, in systems containing an adsorbent, the velocity of enzyme reactions depends upon the concentration of unadsorbed substrate.

Experiments conducted upon the system urease-charcoal-urea confirmed the first postulate, showing that the velocity of decomposition of urea is independent of the presence of an adsorbent, in spite of the fact that 90 % of the urease is adsorbed. Urea causes scarcely any elution of the ferment and the addition of alcohol has little influence upon the velocity of decomposition of urea.

We may hence conclude that in those conditions where an adsorbed enzyme suffers no loss of catalytic power, and the substrate is not adsorbed, the change from a homogeneous to a heterogeneous system, due to the introduction of the adsorbent, does not influence the direction of the reaction, its final equilibrium point, or the velocity of enzymic reaction.

We shall next proceed to examine those systems in which both the enzyme and the substrate undergo adsorption. Such a case is afforded by the systems amylase-dextrin or glycogen-charcoal. Here we find that dextrin and glycogen are strongly adsorbed by charcoal, that the addition of such substances as butyl and propyl alcohols, ether and chloroform greatly diminishes the degree of the adsorption, that amylase is very strongly adsorbed by charcoal and that the addition of alcohols, chloroform or ether, in such quantities as diminish the degree of adsorption of dextrin or glycogen by 50 % does not produce this effect upon amylase. Since the completion of these experiments we have found a confirmation of this fact in a paper published by Unna [1926].

Experiments carried out with the object of comparing the velocity of enzymic hydrolysis in the presence or absence of charcoal show that this velocity increases inversely as the quantity of added adsorbent. In this way, the quantity of glycogen hydrolysed by amylase in the presence of charcoal may be reduced to 5 % of that which would normally be decomposed.

Alcohols added to such systems in such quantities as cause no elution of adsorbed amylase, but only of glycogen or dextrin, cause a commensurate increase in the velocity of hydrolysis of these polysaccharides.

The above results show that, in those cases examined, the cause of differences in the velocity of enzyme reactions lies in adsorption, not of the enzyme, but of the substrate. It would therefore appear that adsorbed enzymes act quite efficiently, and in order to show that this is the case we have been able, making use of the fact that the adsorbed ferment does not undergo elution on treatment with water or glycogen solution, to prove that amylase completely adsorbed on charcoal is capable of hydrolysing glycogen or dextrin.

The next point which we wished to establish was that an adsorbed ferment is as active as when not adsorbed. This would also show that differences in reaction velocity depend, not upon adsorption of ferment, but of substrate. That such is the case was shown by the fact that the velocity of hydrolysis

of glycogen or dextrin in the presence of charcoal depends upon the concentration of unadsorbed substrate, and that where the same quantity of ferment was taken and the concentration of free substrate was the same, the presence of charcoal made no difference to the velocity.

From the results obtained for the system amylase-polysaccharide-charcoal may be drawn the following conclusions. Firstly, adsorbed or free amylase possesses the same activity. Secondly, differences in the velocity of hydrolysis of polysaccharides in the presence or absence of charcoal are due solely to the above described spatial separation of the enzyme from its substrate. This would mean that, whether the enzyme combines chemically with the substrate or whether, as Bayliss [1924, 1925] assumes, an adsorption compound is formed, an adsorbed enzyme is only exceptionally able to enter into contact with an adsorbed substrate. Our figures show that, under these conditions, combination of enzyme with substrate is very limited, if the solution is not stirred, and even with stirring only a minimal combination takes place between the adsorbed enzyme and polysaccharide.

A further extremely important conclusion may be drawn from our experiments, namely, that the law of mass action applies also to enzyme reactions in heterogeneous systems. Up to the present, the equation used to express this law had included the following factors: quantity of enzyme, of substrate and of water. If absolute values be applied in this equation, it is obvious that fundamental differences will exist between ferment systems in the presence or absence of an adsorbent. On the other hand, this need not be the case if not the absolute quantities of the above three components be taken into consideration, but only that part of them which is free to take part in the reaction. In this case, we have shown that the whole of the enzyme present is reactive, whilst, in those experiments in which the whole of the enzyme is adsorbed only the free unadsorbed portion of the substrate is at liberty to enter into chemical or adsorptive combination with it. Hence only this portion takes part in the reaction, and should be exclusively considered in the equation. Introducing this correction, we find that the law of mass action is here followed, and that the velocity of reaction between the whole of the enzyme present and the unadsorbed part of the substrate is independent of the heterogeneity of the system.

Conditions are somewhat more complicated if part of the enzyme is unadsorbed. In this case the possibility would arise that this unadsorbed portion would be free to combine with adsorbed substrate. If as above, we assume that only the unadsorbed fraction of the substrate is free to enter into reaction, and accept this as the active concentration, higher values would be obtained than the theoretical.

Thus we see that an examination of the systems urease-urea-charcoal and amylase-polysaccharide-charcoal yields concurrent results, fully in conformity with our theoretical reasoning. The spatial separation of an enzyme from its substrate by the adsorption of both is a factor inhibitive of enzyme action in heterogeneous systems.

It would be of interest to consider firstly, how general is this phenomenon of the influence of structure, and secondly, whether from the above experiments any other conclusions as to influence of structure on enzyme reactions might be drawn. As to the former problem, Meyerhof and Willstätter and his colleagues [1922, 1925] have shown that adsorbed ferments may act quite normally, without any difference in the degree of their activity.

Hedin's work, however, shows that this fact cannot be generalised, since he found that trypsin and rennin lose their activity on adsorption. We would in this connection emphasise that Willstätter, Waldschmidt-Leitz and Kraut found extremely different degrees of adsorption for the pro- and co-enzymes of trypsin, and it may be that their separation and fixation on the adsorbent leads to the inhibition of their action.

This different behaviour of various enzymes, or even of the same enzyme, using various adsorbents, is probably due to differences in the place of attachment of the enzyme to the adsorbent. Thus it is conceivable that in one case, the adsorbent may be bound to a grouping belonging to the accessory non-characteristic part of the enzyme, whilst in a different case, a group indispensable to the action of the ferment is bound.

Such phenomena as changes in velocity of reaction may, within the living organism, depend not exclusively upon sorption in the most general sense of the word, but may equally well be due to chemical combinations, leading to the local fixation of the enzyme or substrate. In this case, glycogen may enter into some loose combination with some other organic colloid.

Certain analogous phenomena may be produced by mechanical occlusion and precipitation during the passage of some colloid from sol to gel, as exemplified in certain of Henri's [1906] experiments. An enzyme reaction which takes place in a solution of gelatin is inhibited by the presence of gelatin gel.

A certain rôle may also be played here by the degree and condition of saturation of the substrate. Thus, owing to changes in the state of solution of difficultly soluble substances, which readily tend to reprecipitate, their active concentration may vary in spite of the fact that their total concentration remains constant. A number of further possibilities may also exist. Thus certain factors may exist which lead to elution of the enzyme but not of the substrate. Such cases have not so far been noted in the literature of the subject, but are none the less possible. Their effect would be to augment velocity of reaction, since the unadsorbed enzyme would be enabled to react with the substrate.

If we accept the above reservations, and substitute for adsorptive influences all factors tending spatially to separate the enzyme from its substrate, many of the characteristics peculiar to the phenomena of the living cell would be explicable.

A further important conclusion follows from this conception of spatial separation as an inhibitive factor in enzyme reaction. Accepting that in homogeneous systems the total concentrations of enzyme and substrate enter

into the equation expressing the equilibrium constant, whilst in heterogeneous systems, in which the enzyme is almost completely adsorbed, only the concentration of unadsorbed substrate enters, then, for reversibly acting enzymes, the final equilibrium point must be different in these two systems, wherever the synthesised body and its disintegration products are differentially adsorbed.

Let us imagine a reversible reaction in a homogeneous system, where, at the equilibrium point the concentration of synthesised product to disintegration products is as 40 : 60. If the former body is more strongly adsorbed than are the latter, then the presence of an adsorbent would shift the equilibrium point in the direction of synthesis, since in the equilibrium equation would figure concentrations of unadsorbed synthesised product and disintegration products. Since the latter are, in the hypothetical case here considered, less strongly adsorbed than are the former, the proportion of the synthesised product in solution would from the commencement be less than that required for equilibrium, and would remain so throughout the experiment. Equilibrium would supervene when considerably more than 40 % of synthesised substance is present; this increment would be larger, the greater the difference in the degree of adsorption of the substrates. Thus a much greater amount of the body should be synthesised in a heterogeneous than in a homogeneous system, and, on the contrary, a much smaller proportion of the synthesised substance should suffer decomposition under the same conditions. Experiments are now in progress with the object of verifying this hypothesis; these are not yet completed, but we consider that they will in all probability confirm our views.

Should this be the case, this would to a certain extent explain why in intracellular conditions the equilibrium point of enzymic hydrolyses lies on the side of synthesis. In such cases as in the synthesis of polysaccharides or proteins, the end-products of reaction are strongly adsorbed, whilst their progenitors or decomposition products are considerably less so.

Experimental.

Solutions of urea and of urease, or of both of these substances together, were added to charcoal, equal proportions of the two former substances taken separately without charcoal, and the amounts of urea converted in both cases were compared. The solutions were filtered either at the beginning of the experiments or after certain intervals of time. The results shown (Tables II to V) are derived from experiments in which both series were carried out simultaneously, using charcoal taken from the same stock. The urease taken was urease powder, purchased from Squibb, a fresh solution being prepared for each series of determinations. In this way, the substances used in each series, both as regards enzyme and adsorbent, were as far as possible uniform.

Urea-ammonia was determined by Schlösing's or Folin's method at a point at which not more than 15 % of the urea present was decomposed. The quantity of urea adsorbed on the charcoal was taken as the difference between that added and that found in the filtrate.

Table II. *Percentage adsorption of urea measured in 2 cc. of solution.*

Amount charcoal added g.	No. of experiments	% urea	% of original urea adsorbed	
			Average	Deviations
0·2	4	0·2	2·4	2·2–2·8
0·4	4	0·4	4·6	3·9–4·8

Table III. *Mg. urea decomposed in the filtrate. Average of five experiments.*

Urease without charcoal	Urease with charcoal	Urease with charcoal and butyl alcohol	Urease with charcoal and propyl alcohol
20·0	0·1	0·1	0·3
19·2	0·3	0·2	0·2

Table IV. *Mg. of urea decomposed.*

Urea, charcoal and urease left in contact without filtration		Urea, charcoal and urease with filtration		Urease and urea		Urease and charcoal filtered and urea added	
0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea
11·8	19·9	3·5	4·8	11·9	20·2	0·0	1·5
11·6	19·8	3·3	5·1	11·5	19·9	0·9	1·0

Table V. *Mg. of urea decomposed.*

Series	Time (hours)		0·1 g. urease, 0·5 g. charcoal and 20 cc. 0·2 % urea					Urease + 0·2 % urea				
			I	II	III	IV	V	I	II	III	IV	V
α	2	No alcohol	5·3	5·5	6·6	5·4	2·7	5·1	5·0	—	5·6	2·8
		Propyl alcohol	—	—	—	—	—	5·1	5·1	—	—	2·8
		Butyl alcohol	—	—	—	—	—	5·0	5·3	—	—	2·7
	4	No alcohol	10·4	10·8	13·1	10·5	5·5	10·2	10·2	13·5	10·7	5·7
		Propyl alcohol	—	—	14·9	11·0	5·7	14·0	10·4	13·5	10·9	5·6
		Butyl alcohol	—	—	15·1	10·8	5·4	—	10·1	13·5	10·7	5·4
β	2	No alcohol	5·2	5·6	6·1	5·7	2·8	5·1	5·2	—	5·8	2·4
		Propyl alcohol	5·3	5·7	—	5·9	2·8	5·2	5·3	—	—	2·3
		Butyl alcohol	5·3	5·4	—	5·9	2·9	5·1	5·3	—	—	2·4
	4	No alcohol	10·5	10·4	12·1	10·7	5·7	10·1	—	11·8	10·8	5·0
		Propyl alcohol	10·7	—	13·9	11·0	5·8	10·2	—	11·8	11·0	5·0
		Butyl alcohol	10·6	—	14·1	11·0	5·8	10·2	—	11·5	10·9	4·9
			0·1 g. urease, 0·5 g. charcoal and 20 cc. 0·4 % urea					Urease + 0·4 % urea				
α	2	No alcohol	10·6	11·4	—	11·7	—	10·2	10·5	—	11·1	5·9
		Propyl alcohol	11·0	11·5	—	11·7	—	10·3	11·2	—	11·3	5·8
		Butyl alcohol	10·8	11·4	—	11·9	—	10·6	—	—	11·1	5·8
	4	No alcohol	21·1	22·7	—	21·2	—	21·0	20·3	—	21·1	11·9
		Propyl alcohol	21·4	23·0	—	21·4	—	20·3	—	—	21·4	12·0
		Butyl alcohol	21·5	22·8	—	21·4	—	22·5	—	—	21·0	12·0
β	2	No alcohol	10·4	—	—	11·6	—	10·6	11·0	—	11·4	5·6
		Propyl alcohol	10·7	—	—	11·8	—	—	—	—	11·2	—
		Butyl alcohol	10·7	—	—	11·7	—	—	—	—	11·6	—
	4	No alcohol	20·7	22·0	—	21·4	11·2	20·9	21·6	—	22·1	11·3
		Propyl alcohol	—	22·1	—	22·0	—	21·0	22·5	—	22·1	11·3
		Butyl alcohol	—	22·0	—	21·8	—	20·8	21·8	—	22·3	11·2

The second type of experiments was carried out on amylase and dextrin or glycogen. The enzyme used was purified amylase, purchased from Merck;

in some cases the ferment contained in frog's blood was taken, proceeding according to Lesser [1924]. Here too, determinations were made at such intervals that up to 15 % of the polysaccharides taken had been decomposed. The degree of hydrolysis of dextrin was estimated by Bertrand's method. The amount of polysaccharides adsorbed by charcoal was determined by the hydrolysis of the filtrate.

A. *The system urease-urea-charcoal.*

1. *Adsorption of urea.* The values shown in Table II indicate that the specimen of charcoal used adsorbs urea very feebly.

2. *Adsorption of urease.* 0·5 g. of charcoal was added to solutions containing 0·1 g. of urease, the temperature of the solutions being 20°. After 15–30 minutes, the solutions were filtered, the residue was washed with two 5 cc. portions of water, and 0·2 g. of urea added to the filtrate, so that the latter is a 0·4 % solution of urea. Simultaneously an equal quantity of urea was added to a second, also filtered, solution of urease, without previous charcoal treatment. The results obtained, given in Table III, show that in these experiments 85–95 % of the enzyme present undergoes adsorption by charcoal.

The adsorption of urease in the presence of butyl and propyl alcohols was next studied. The results obtained show that neither of these alcohols is able either to cause elution of adsorbed urease, or to diminish the degree of adsorption of the latter when added simultaneously with it (Table III).

3. *Activity of adsorbed urease.* The degree of adsorption of urease was found to be unaffected by the subsequent addition of urea. This fact is of great importance for demonstrating that adsorbed urease retains its enzymic properties, and that the conditions under which free urease in a homogeneous system and adsorbed urease in a heterogeneous system are examined are strictly comparable.

1 cc. of 0·2 % urease solution was added to 10 cc. water containing 0·2–0·5 g. of charcoal. After 30–60 minutes, half of the solution was filtered and urea solution added to both portions, the second portion being also filtered after from 5 to 10 minutes. The amount of unadsorbed urease was estimated by determining the amount of urea decomposed in the first filtrate within two hours. Since the concentration of urea was the same in all cases, differences in the final concentration could arise only from different urease contents. The values given in Table IV show a certain difference between those experiments in which urease was added before and after filtration. These discrepancies are probably largely due to the urea being in contact with the enzyme before filtration. A further cause of these differences lies, however, in the slight elution of urease by urea, as is shown by those experiments in which the quantity of ammonia formed in two hours by filtered urease solutions to which urea is added, is compared with the differences between the amount of ammonia formed in two hours after filtration of a similar solution containing urea, and that present immediately after filtration.

Having thus shown the degree of adsorption of urease by charcoal to be extremely high, and that urea has scarcely any elutive effect, it became possible to investigate the question of the efficiency of adsorbed urease for the reaction of the decomposition of urea.

The velocities of decomposition of urea in the system urease-urea-charcoal, with and without shaking, were compared, and found to be identical. Hence, it would follow that the efficiency of action of urease is independent of its distribution throughout the solution.

B. *The system polysaccharide-amylase-charcoal.*

1. *Adsorption of polysaccharide.* 0·2 or 0·4 g. of charcoal was added to 20 cc. of a solution of dextrin or glycogen, which was filtered after a lapse of 30 minutes. Table VI shows that, in those cases where 0·2 g. of charcoal was added, on the average 50·5 % of the dextrin was adsorbed, whilst with 0·4 g. of charcoal the degree of adsorption was considerably greater, on the average 72·8 %. Glycogen is adsorbed to a slightly greater extent, the average adsorption in a solution containing 0·4 g. of charcoal being 74 %.

The adsorption of the above substrates is therefore quite considerable, as only about 20 % of the given polysaccharide can exist in solution. Using smaller concentrations of substrate, even greater differences between the total content of the latter and its concentration in solution were obtained.

Table VI. *Mg. reducing sugars found after hydrolysis.*

Compound	No. of experiments	In absence of charcoal	0·2 g. charcoal	0·4 g. charcoal
Dextrin	7	Average 13·5	Average 6·7	Average 3·7
		Deviations 13·1–14·0	Deviations 6·3–7·2	Deviations 3·3–4·6
Glycogen	4	Average 14·1	—	Average 3·4
		Deviations 14·1–14·2	—	Deviations 3·2–3·8

Table VII. *Mg. reducing sugars found after hydrolysis. Averages.*

Compound	No. of experiments	Without charcoal	With charcoal		With charcoal and alcohols added		Alcohol added
			0·2 g.	0·4 g.	0·2 g.	0·4 g.	
Dextrin	12	12·2	6·9	3·2	8·5	4·7	Butyl; propyl
Glycogen	4	14·2	—	3·6	—	4·5–4·9	Butyl; propyl

Table VIII. *Mg. reducing sugars found after hydrolysis. Average figures for six experiments.*

Without charcoal	0·4 g. charcoal added	0·4 g. charcoal and various other substances	Other substances added
12·8	3·3	7·0	Amyl alcohol
12·0	2·9	7·6	Chloroform
12·6	3·4	8·2	Ether

In the presence of bodies strongly adsorbed by charcoal, such as propyl or butyl alcohol, considerable elution of glycogen or dextrin took place (see

Table VII). The behaviour of systems containing glycogen is analogous, as may be seen from the results of two experiments conducted on this system, and probably all polysaccharides behave in this way.

The action of amyl alcohol, of ether and of chloroform is, as we see from Table VIII, similar to that of the above alcohols. Thus it appears that more strongly adsorbed substances, such as narcotics, lead to the elution of adsorbed polysaccharides. By making use of this important fact we can change at will the concentration of dissolved polysaccharide without affecting the total amount present.

In all probability, the above observation is of considerable significance in physiological reactions. A second perhaps equally important conclusion which may be drawn from this observation is that the degree of elution of polysaccharides by alcohols is proportional to the degree of adsorption of the former.

2. *Adsorption of amylase by charcoal.* The enzyme-adsorbent system was filtered, a solution of dextrin added to the filtrate, and the whole left at 37° in a thermostat for 7–8 hours. For experiments in aqueous solution, 10 cc. of 1 % amylase purchased from Merck and 0·5 g. of charcoal were taken. After filtration, 10 cc. of 1 % dextrin were added to the filtrates (Table IX). Over 90 % of the amylase present undergoes adsorption, and is present in suspension as an adsorption compound.

From Table X we see that the degree of adsorption of amylase is unaffected by the addition of such powerful elutive agents as butyl and propyl alcohols. Similar results were obtained using diastase from frog's blood. Here a slightly different experimental procedure was adopted. Instead of filtration, the solutions were centrifuged. Almost identical results were obtained with those mentioned above for Merck's amylase, *i.e.* strong adsorption, without elution by alcohols. The only difference is in the slightly lower degree of adsorption of frog amylase.

Table IX. *Mg. dextrin decomposed by filtered amylase solutions. Average figures for eight experiments.*

Without charcoal		With 0·5 g. of charcoal	
Average	Deviations	Average	Deviations
72·1	69·7–74·2	6·8	6·6–7·1

Table X. *Mg. of dextrin decomposed by filtered solutions of amylase. Average figures and deviations for six experiments.*

1	2	3	4	5	6	7	8
Without charcoal	0·5 g. charcoal	Charcoal and propyl alcohol	Charcoal and butyl alcohol	Propyl alcohol	Butyl alcohol	Charcoal filtered and propyl alcohol added to filtrate	Charcoal filtered and butyl alcohol added to filtrate
70·6	6·8	6·7	6·7	69·6	69·2	6·6	6·7
68·7–73·0	6·7–7·1	6·6–6·9	6·6–6·9	67·8–71·8	67·8–72·3	6·5–6·8	6·5–6·8

Differences in the velocity of hydrolysis of dextrin.

We have shown that the enzymic hydrolysis of dextrin proceeds in every case more rapidly in a homogeneous than in a heterogeneous system. The retardation of reaction increased with the amount of adsorbent added, until finally only 5 % of the hydrolysis taking place in a homogeneous system was observed.

Two factors might lead to this retardation: the enzyme might be inactivated by adsorption or the enzyme might be spatially separated from its substrate by the fixation of both on the adsorbent. Since we have evidence that adsorbed enzymes may still be active, it remained to establish which factor or factors enter into the present case. It appeared that in order to elucidate this point, certain properties of the enzyme in question would have to be considered. With this end in view, three types of experiments were undertaken. These had as their object to establish, firstly, that the addition of alcohols in such quantities as cause elution of substrate but not of ferment accelerates enzymic hydrolysis; secondly, that completely adsorbed amylase can catalyse the decomposition of dextrin; and thirdly, that the velocity of enzymic hydrolysis is proportional to the concentration of unadsorbed dextrin in solution, and is equal to that of the same concentration of dextrin in the presence of the same quantity of ferment in a homogeneous system.

In our experiments upon the influence of alcohol, the concentrations of substrate, ferment and charcoal were the same as before. Four determinations were made of hydrolysis in the systems charcoal-amylase-dextrin and amylase-dextrin, with and without the addition of butyl and propyl alcohols in such concentration as to cause no elution of the ferment.

Table XI. *Mg. of polysaccharide decomposed. Average figures.*

			1 % amylase-charcoal-polysaccharide				
Substrate	No. of experiments	Time hours	Without alcohol	Butyl alcohol	Propyl alcohol	Charcoal g.	Amylase-polysaccharide
Dextrin	3	6	32·1	47·8	46·1	0·2	55·5
	3	6	16·1	28·1	26·4	0·4	55·2
	3	12	63·9	79·0	76·6	0·2	102·7
	3	12	24·1	36·3	37·2	0·4	103·4
Glycogen	2	6	10·8	16·7	17·1	0·4	46·9
	3	6	26·7	31·2	33·0	0·2	44·7
	3	12	19·9	32·0	31·8	0·4	80·2

The results shown in Table XI indicate that the addition of alcohols considerably accelerates the velocity of hydrolysis of polysaccharides. This effect can be due only to an increase in the concentration of unadsorbed dextrin. It hence follows that, in the presence of substances which adsorb both enzyme and substrate, the addition of alcohols or other narcotics leads to an augmentation of the velocity of reaction due to elution of the substrate, but not of the ferment.

It remains to be proved, however, whether the adsorbed ferment has any hydrolytic action upon dextrin. Thus, in the above case, the possibility may

exist that, both in the presence or absence of alcohols, only the unadsorbed portion of the enzyme has any action, differences in velocity of hydrolysis being due exclusively to elution of substrate. We see, however, no *a priori* reason for believing that adsorption should inactivate an enzyme, unless, of course, attachment to the adsorbent binds some group indispensable to the action of the ferment.

In order, however, to establish that amylase remains active after adsorption, the following experiments were conducted. 1 g. of charcoal was added to 10 cc. of 0·5 % amylase, and the mixture shaken mechanically at 25° for two hours, after which it was centrifuged and the supernatant liquid completely poured off. The residue was then washed five times with water at 25°, glycogen solution was added, and the whole left for from 2 to 4 hours at 37°. We know from Unna's experiments that under these conditions adsorbed amylase is not eluted by glycogen, so that any hydrolytic action observed must be due solely to the adsorbed ferment. Table XII shows that the hydrolytic action of such enzyme-adsorbent preparations upon glycogen is quite considerable.

Table XII. *Non-elution of amylase by polysaccharides.*

Substrate	No. of experiments	Mg. reducing sugars found after contact of from 2–8 hours of adsorbed ferment with substrate		Mg. reducing sugars produced in solution after centrifuging and decanting
		Average	Deviations	
Dextrin	6	21·8	10·6–31·5	0
Glycogen	4	26·3	11·5–33·2	0

That longer contact of adsorbed amylase with glycogen solution does not lead to elution of ferment is shown by the following type of experiment.

The concentration of reducing sugars found after a 4–8 hours' contact of glycogen solution with the charcoal-amylase preparation is determined, and the mixture centrifuged. A portion of the clear supernatant liquid exhibits no increment in the amount of reducing sugars present after a further lapse of 6–8 hours (cf. Table XII).

We may hence consider the activity of adsorbed amylase to be conclusively established, as also its non-elution by glycogen solutions.

The above result is, however, only a proof that adsorbed amylase possesses enzymic activity; whether this activity is as great as that of the free ferment remains to be proved.

Thus the retardation of hydrolytic action in systems of the type amylase-charcoal-polysaccharide may be ascribed either to depression of enzyme action due to adsorption, or to diminution in the concentration of free substrate owing to its fixation on the adsorbent. With a view to the elucidation of this question, the following experiments were carried out.

Charcoal and dextrin solution were added to a solution of amylase at 0°, and after 20 minutes the mixture was filtered, and the dextrin content of the filtrate determined. Two further portions were then prepared, one of

which was identical with the above, whilst the other from which charcoal was absent contained an identical concentration of amylase but a concentration of dextrin equal to that found in the filtrate in the preliminary experiment. Both portions were placed in a thermostat and their reducing sugar content was determined after from 2 to 8 hours.

Concordant results were obtained from a number of experiments in which the velocity of hydrolysis was equal in both cases (cf. Table XIII). It hence follows that the efficiency of adsorbed amylase is quantitatively equal to that of the free enzyme.

Differences in the velocity of hydrolysis of polysaccharides by amylase, caused by the addition of charcoal to the system, can therefore be ascribed solely to the spatial separation of the enzyme from its substrate by this fixation on the adsorbent.

Table XIII.

	Amylase-charcoal-dextrin		Amylase-dextrin	
No. of experiments	Average % dextrin unadsorbed	Average glucose found mg.	Average % dextrin	Average glucose found mg.
4	0·71	31·2	0·71	32·0
4	0·53	18·8	0·53	18·9
4	0·29	6·2	0·29	6·1
4	0·12	3·7	0·12	3·6

As to the question of the applicability of the law of mass action, it follows that the velocity of enzyme reaction is, within certain limits, independent of the absolute concentration of the substrate, but is proportional to that of the unadsorbed substrate. For this reason the velocity of reaction diminishes proportionately to the amount of adsorbent added (Table XIII).

Summary.

1. The effect of heterogeneity of the medium upon the course of enzymic reactions is considered in relation to reactions in the living organism and is studied experimentally upon models consisting of enzyme, adsorbent and substrate.

2. In the case of the system urease-charcoal-urea, urease is shown to be almost quantitatively adsorbed, whilst urea is adsorbed only to a very small extent. The addition of propyl or butyl alcohol produces no measurable elution of the ferment.

3. In such a system, in which the ferment, but not the substrate, is adsorbed, the velocity of enzymic reaction is practically the same as in the absence of an adsorbent, and is unaffected by the addition of narcotics.

4. In the case of the system amylase-charcoal-glycogen or -dextrin, the ferment is, like urease, practically quantitatively adsorbed, whilst the percentage adsorption of dextrin or glycogen amounts to from 50 to 75 %, depending upon the quantity of charcoal added.

5. The addition of propyl or butyl alcohol, or of other narcotics, leads to an elution of about 50 % of the substrate; at the same time no measurable elution of ferment takes place. The percentage elution of polysaccharide by alcohols is greater, the greater the percentage adsorption.

6. In this system, in which both enzyme and substrate undergo adsorption, the velocity of enzymic hydrolysis is considerably lower than in the absence of an adsorbent.

7. This diminution in reaction velocity is explained as being due to the spatial separation of enzyme and substrate by fixation on different parts of the adsorbent surface.

8. The addition of narcotics to this system accelerates the reaction by increasing the concentration of unadsorbed substrate, which alone is able to enter into reaction.

9. The law of mass action is followed in enzyme reactions in a heterogeneous medium, with the reservation that the effective concentration of the substrate be taken as that part of the latter which is in solution.

10. The non-degradation of glycogen by frog's liver during the winter months is explained on the basis of the above findings, amylase and glycogen being supposed to be fixed on different parts of the structural surface of the liver cell.

REFERENCES.

Bayliss (1924). Principles of general physiology (London).
—— (1925). The nature of enzyme action (London).
Hedin (1915). Grundzüge der physiol. Chemie (Wiesbaden).
Henri (1906). *Compt. Rend. Acad. Sci.* **142**, 97.
Hofmeister (1901). Die chemische Organisation der Zelle (Brunswick).
Josephson (1925). *Z. physiol. Chem.* **147**, 1.
Lesser (1920). *Biochem. Z.* **102**, 294.
—— (1924). *Biochem. Z.* **140**, 397.
Meyerhof (1914). *Arch. ges. Physiol.* **157**, 251.
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Przylecki (1922). *Arch. int. Physiol.* **20**, 144.
Unna (1926). *Biochem. Z.* **172**, 392.
Willstätter and Kraut (1925). Cf. Oppenheimer, "Die Fermente," vol. 1 (Leipzig).
Willstätter, Waldschmidt-Leitz and Memmen (1922). *Z. physiol. Chem.* **125**, 93.

CXXXVII. STUDIES IN PURINE METABOLISM.

III. BASAL METABOLISM AND PURINE CONTENT.

By RICHARD TRUSZKOWSKI.

From the Biochemical Laboratory, Faculty of Veterinary Medicine, Warsaw University.

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Basal metabolism has been found experimentally to be more nearly a function of body surface than of weight; this relation, the law of surfaces, is, as has been shown by Rubner [1883], Richet [1891], Frank and Voit [1901], Slowtzoff [1903], Lautanié [1905] and Benedict and Talbot [1915], only approximately exact. Further, since at thermal neutrality basal metabolism still remains a function of body surface, it would appear that thermolysis is not the cause of this constancy, but that this must be ascribed to some other factor. Very numerous explanations of the law of surfaces have been advanced. These may be divided into four groups—anatomical, physico-chemical, nervous and chemical. The first of these groups, represented by von Hösslin [1888], Terroine [1924, 1], and others, connects basal metabolism with the volume of blood passing a given area in unit time. The second group [Pfaundler, 1916, 1921] considers the cellular surface to be the deciding factor in thermogenesis, although Rubner's [1913] findings tend to discredit this view. The third group [Richet, 1889; Lefèvre, 1911; de Almeyda and de Fialtro, 1925] consider basal metabolism to be under nervous control.

The fourth group deals with chemical differences, and is mainly occupied by attempts at determining the "active mass" of an animal. According to these views, some portion of the complex chemical mechanism of the animal system is responsible for the energy output of the latter, the remainder serving various ends, such as structural, connective, and reserve tissues. If, therefore, the weight of this active portion of the animal could be determined, this should stand in some simple proportion to its basal metabolism.

Attempts at determining this active mass have been so numerous that I shall here cite only the more important papers on the subject. According to Terroine [1924, 2] neither the lipoid content, nor that of lipoid phosphorus can in any way be connected with basal metabolism. As to the mineral constituents, he finds that neither their total amount, nor the percentage of iron, which might be supposed to catalyse oxidations, can be considered to be the active mass, and in the same paper he states that there is no real reason for assuming purine content to be the active constituent in question, and concludes

that, since thermal neutrality is a condition almost never met with under natural conditions, the law of surfaces is nevertheless valid and rational and that it is thermolysis which determines thermogenesis. In certain other papers, however [Terroine and co-workers, 1923, 1924, 1926], the opinion is put forward that the active mass of the body may be expressed by its protein content. Since this is proportional to the body weight only in animals divested of reserves of all types, this type of metabolism, where the energy output is constant per unit weight of animal, is met with only in starvation. Hence, Terroine, Feuerbach and Brenckmann [1924] concluded that the average animal contains two types of material; one a vital, active portion, of constant composition, unalterable by the exigencies of life, and the other, consisting of reserves, whose relative proportion is variable and which normally mask the active mass of the animal. In 1923, however, the same authors found that, since the proportion of protein present in animals so different as mice and bulls differs very little, whilst the basal metabolism per unit mass differs very widely, the protein content cannot be considered to be representative of the active mass.

Le Breton [1926] is, however, of the opinion that body-proteins are divisible into two classes—active protoplasm and inactive protoplasm, or paraplasm. According to this hypothesis, the size of an animal is determined by the inherent metabolic intensity of its active protoplasm, whose heat production must be equal to loss of heat from body-surfaces. If, by the inclusion of paraplasm in the cytoplasm the animal can dilute its active mass, it can then grow larger until thermal equilibrium is attained.

Terroine and Roche [1925], however, conclude from measurements of the intensity of respiration of minced tissues of homeotherms of different sizes that the metabolic intensity of protoplasm is identical in the animals studied. These authors therefore concluded that the intensity of basal metabolism depends, not upon differences in protoplasm, but upon blood supply.

Terroine, in one of the above cited papers [1923] expressed the opinion that nuclear matter is the active mass in question. Indeed, a number of biologists have drawn attention to the importance of the nucleus in cellular metabolism. Thus, Palladine [1896] considered that the respiration of plant tissues is proportional to their nuclear content, Loeb [1913] and Osterhout [1917] found that the nucleus is the seat of the most intensive oxidative processes taking place in living tissues, and Robertson [1923] thought that oxidative activity should be proportional to the nuclear-plasmic ratio of a given tissue or cell. Warburg [1909] showed that nucleated blood corpuscles consume more oxygen than non-nucleated, and that, further [1912], if a cytolysed suspension of avian erythrocytes be centrifuged, respiration is confined to the lower layer, in which may be found the nuclei. On these grounds, therefore, one might expect the basal metabolism of an animal to be proportional to the nuclear content of the latter.

In our opinion, the magnitude of the nuclear-plasmic ratio would be an

even better index of metabolic intensity than merely the nuclein content, since it deals with only the actively reacting portions of the cell, excluding differences due to variable water and reserve contents among the various individuals examined.

Since we have been able to find no reliable values for the purine content or nuclear-plasmic ratio of various animals, this research was undertaken with a view to the confirmation or otherwise of the above hypothesis, connecting basal metabolism with purine content or nuclear-plasmic ratio.

Experimental.

Purine-nitrogen, which is taken as representative of nuclear content, the total nitrogen, and the nuclear-plasmic ratio were determined by the methods described in a previous paper [Truszkowski, 1926]. The animals examined were rats, guinea-pigs, rabbits, dogs, horses and cattle. The first two species were analysed whole, after removal of food remains from the alimentary tract and mincing. In the case of rabbits, dogs, horses and cattle, skeletal muscle and liver alone were analysed, since for technical reasons the analysis of whole animals larger than guinea-pigs presented too great difficulties: for comparative purposes, analyses were also made of the skeletal muscle of rats. It is realised that results obtained for one portion of an animal need not necessarily apply to the animal as a whole, but in view of the preponderance of muscular and glandular tissue among the nitrogenous constituents of the homeotherms, there is some justification for considering that the values obtained for muscle and liver are to a large extent representative of those which would be obtained for the entire animal.

The values for whole rats are taken from our previous paper (Part I, p. 441), and the mean values are given in Table II. Those obtained for skeletal muscle taken from six rats are shown in Table I. The water content, and total and purine-nitrogen are higher than for the whole animal, but the nuclear-plasmic ratio differs by less than 3 %. In the same table may be found the results obtained for guinea-pigs. Here the solid substance varies from 28 to 29·8 %, total nitrogen from 3·421 to 3·529 %, and purine-nitrogen from 72·18 to 77·47 mg. %; the nuclear-plasmic ratio varies from 21·56 to 23·63, on the average $22{\cdot}60 \times 10^{-3}$.

The values for skeletal muscle and liver of rabbits are taken from some unpublished results, which Dr Dmochowski has communicated to me. As these will be published separately, and deal with a different problem, only the mean values will be given here (Table II), and the same applies to those obtained by Miss Rowinska and Mr Zdunkiewicz for cattle and by myself for dogs. In the latter case, the animals weighed from 5 to 10 kg. and were fed on a diet poor in protein (oatmeal, potatoes, etc.).

In the case of horses (Table I) fairly wide variations were found; for muscle, total nitrogen varies from 3·119 to 3·550%, calculated for dry tissue from 13·32 to 15·40 %; purine-nitrogen is from 77·0 to 83·7 mg. %, and the nuclear-

plasmic ratio varies from 22·17 to 26·48 $\times 10^{-3}$. The liver has a lower water content (71·1 %) due obviously to the presence of some non-nitrogenous substances, since the nitrogen content is lower than in muscle, being 3·204 and 11·15 % for fresh and dry weight respectively. The purine content is considerably higher than for muscle, and is, on the mean of two determinations, 147 mg. %, and the nuclear-plasmic ratio, which shows wide variations for liver tissue in general, is in one case 42·12 and in the other 54·89, on the average 48·50 $\times 10^{-3}$.

Table I.

Species	No.	Material taken	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio
Rat	1	Skeletal muscle	28·0	3·850	13·90	81·7	294·9	$21·68 \times 10^{-3}$
,,	2	,, ,,	28·0	3·842	13·80	78·2	282·2	$20·76 \times 10^{-3}$
Guinea-pig	1 ♂	Whole	29·8	3·447	11·67	80·3	269·5	$23·63 \times 10^{-3}$
,,	2 ♂♀	,,	28·0	3·529	12·57	77·5	276·7	$22·51 \times 10^{-3}$
,,	3 ♂♀	,,	28·0	3·421	12·22	72·2	257·7	$21·56 \times 10^{-3}$
Horse	1 ♀	Skeletal muscle	20·8	3·188	15·32	—	—	—
,,	2 ♂	,, ,,	24·9	3·550	14·26	77·0	309·2	$22·17 \times 10^{-3}$
,,	3 ♂	,, ,,	23·4	3·119	13·32	—	—	—
,,	4 ♂	,, ,,	23·2	3·142	13·54	81·0	349·3	$26·48 \times 10^{-3}$
,,	5 ♀	,, ,,	21·8	3·357	15·40	83·7	383·8	$25·57 \times 10^{-3}$
,,	6 ♀	,, ,,	21·8	3·342	15·30	81·9	375·8	$25·14 \times 10^{-3}$
,,	3 ♀	Liver	30·3	2·987	9·86	155·4	513·1	$54·89 \times 10^{-3}$
,,	4 ♂	,,	27·5	3·421	12·44	138·3	502·9	$42·12 \times 10^{-3}$

The mean values obtained by other authors and by myself are given in Table II. Rats and guinea-pigs give fairly concordant values, all figures obtained for the latter being slightly higher than for the former, with the exception of the percentage of solid substance. Thus, for whole animals, it would not appear that either purine content or nuclear-plasmic ratio is in any way connected with basal metabolism.

For muscular tissue much more divergent results are found.

Table II. *Mean values.*

Species	Material taken	% solid substance	% N live weight	% N dry weight	Purine-N. mg. % live weight	Purine-N. mg. % dry weight	Nuclear-plasmic ratio	Author
Rat	Whole	33·45	3·285	9·745	65·2	196·0	$20·62 \times 10^{-3}$	Truszkowski
Guinea-pig	,,	28·6	3·466	12·152	76·7	268·0	$22·60 \times 10^{-3}$	Truszkowski
Rat	Skeletal muscle	27·7	3·846	13·85	80·0	288·6	$21·22 \times 10^{-3}$	Truszkowski
Rabbit	,, ,,	24·5	3·777	15·41	115·5	471·3	$31·54 \times 10^{-3}$	Dmochowski
Dog	,, ,,	21·2	2·514	12·06	54·2	264·0	$22·01 \times 10^{-3}$	Truszkowski
Horse	,, ,,	22·6	3·283	14·53	80·9	358·8	$25·33 \times 10^{-3}$	Truszkowski
Cattle	,, ,,	25·3	3·933	15·58	76·5	284·1	$19·86 \times 10^{-3}$	Rowinska and Zdunkiewicz
Rabbit	Liver	26·3	2·819	9·85	140·0	536·3	$52·25 \times 10^{-3}$	Dmochowski
Dog	,,	24·4	2·335	9·65	86·7	355·5	$40·53 \times 10^{-3}$	Truszkowski
Horse	,,	28·9	3·204	11·15	146·9	508·0	$48·50 \times 10^{-3}$	Truszkowski
Cattle	,,	24·8	3·115	12·56	118·8	478·9	$39·71 \times 10^{-3}$	Zdunkiewicz

No proportionality between size of animal and water, nitrogen, or purine content of muscles, or of their nuclear-plasmic ratio, appears to exist. Thus, whilst rat muscle has the highest percentage of solid substance, that of cattle

is the next largest, then that of rabbits, horses and dogs. The nitrogen content decreases in the order—cattle, rats, rabbits, horses, and dogs; and the purine content in the order—rabbits, horses, rats, cattle and dogs. The nuclear-plasmic ratio is highest for rabbits, *i.e.* $31{\cdot}54 \times 10^{-3}$, then horses, dogs, rats and cattle, $19{\cdot}86 \times 10^{-3}$. Thus we see that rats and cattle, whose average weights are as 1 : 2500 are the most similar to each other in purine and nitrogen content of muscle, and that generally, no regularity exists in the values obtained for the above series of animals.

The same may be observed in the values found for the liver. Here horses have the highest percentage of solid substance, next rabbits, cattle and dogs. The highest nitrogen content may be found in horses, then cattle, rabbits and dogs, whilst the purine nitrogen content decreases in the order—horses, rabbits, cattle and dogs. The nuclear-plasmic ratio is, as for muscle, highest in the rabbit ($52{\cdot}25 \times 10^{-3}$), then follow horses, dogs and cattle ($39{\cdot}71 \times 10^{-3}$). Here, also, there is no connection between the values obtained and the weight, *i.e.* the basal metabolism of the animal.

It is, however, interesting that the liver has a nuclear-plasmic ratio approximately double that of skeletal muscle, and this is in accordance with histological findings for the same tissues. No particular stress is laid upon the values other than this ratio, since it is realised that these depend upon the state of nutrition of the given animal at the moment when killed, and to a certain extent upon the previous history of the latter, which is, in the case of horses and cattle, unknown.

Discussion.

The results given above show that considerable variations may exist in the nitrogen and purine contents of the liver and skeletal muscle of various animals and in their nuclear-plasmic ratios, but that these variations are in no way connected with the basal metabolism of the given animal. The values obtained are, however, to a certain extent constant for a given species, and appear, further, to be characteristic of the particular organ examined.

There exists, however, the possibility that, whilst basal metabolism does not depend upon nuclear mass, it may depend upon the active surface of the nucleus. Thus, admitting that reactions of oxidation take place within the nucleus, their intensity will depend not so much upon its mass as upon the nuclear-plasmic intersurface. It is therefore conceivable that equal nuclear masses need not be equivalent, their activity depending upon the development of their surfaces. This, again, may be influenced by two factors, namely, cell dimensions and concentration of active substances within the nucleus.

In the first case, it is obvious that the smaller the cell, the greater the relative nuclear surface, assuming the volumes of nucleus and plasma to diminish proportionally. Unfortunately very little work has been done in this field, and those values which I have been able to find are contradictory. Amelung [1893] and Strasburger [1893] found that cells of the same organ in

plants of different size but of the same species are of equal size. Rabl [1899] found that the dimensions of epithelial cells and lens fibres were the same for dogs of various sizes. On the other hand, Levi [1905], found that sections of cerebral cells of different mammals were greater, the greater the weight of the animal, that of the ox being 104·3μ, whilst that of the mouse is 37·25μ, and differences of the same order may be found for lens fibres. These differences are, however, insufficient to explain differences in basal metabolism, since, if we assume that the nuclei diminish in radius to the same degree as the cells, this would mean that the surface of unit weight is approximately three times greater for mouse than for ox nuclei, whereas the basal metabolism of the former animal is about 20 times as great as for the latter. However, in view of the lack of reliable data on this point, it is unprofitable further to discuss it, and the same applies to the possibility of enlargement of nuclear surface by dilution.

SUMMARY.

1. The chemical nuclear-plasmic ratios of whole rats and guinea-pigs differ little from one another.

2. The values obtained for purine- and total nitrogen, and for the nuclear-plasmic ratios of liver and of skeletal muscle of rats, rabbits, dogs, horses and cattle vary within wide limits, but are in no way connected with the basal metabolism of the animals in question.

3. The purine content of animals cannot therefore be identified with their "active mass."

I wish to express my sincere gratitude to Prof. St. J. Przylecki, who proposed the above research, and to whom I am indebted for constant help and advice.

REFERENCES.

de Almeyda and de Fialtro (1925). *Compt. Rend. Soc. Biol.* **92**, 230.
Amelung (1893). Cited from J. Bury, "Kosmos" (Lwow, Poland), 1910, p. 899.
Benedict and Talbot (1915). *Carnegie Inst. Washington, Rep.* No. 231.
Frank and Voit (1901). *Z. Biol.* **42**, 309.
von Hösslin (1888). *Arch. Anat. Physiol.* p. 323.
Lautanié (1905). Eléments de Physiologie, 2nd edit. (Paris), p. 570.
Le Breton (1926). *Ann. Physiol.* **2**, 606.
Lefèvre (1911). Chaleur animale et bioenergetique (Masson, Paris).
Levi (1905). *Anat. Anzeig.* (Ergänz. Heft), **27**, 156.
Loeb (1913). Artificial parthenogenesis and fertilisation (Chicago).
Osterhout (1917). *Science*, **46**, 367.
Palladine (1896). *Rev. Bot.* **18**, 225.
Pfaundler (1916). *Z. Kinderheilk.* **14**, 48.
—— (1921). *Arch. ges. Physiol.* **188**, 273.
Rabl (1899). *Z. wiss. Zool.* **67**, 135.
Richet (1889). Le chaleur animale (Alcan, Paris).
—— (1891). *Arch. Physiol. Norm. Path.* **23**, 74.
Robertson (1923). The chemical basis of growth and senescence (Philadelphia), p. 193.

Rubner (1883). *Z. Biol.* **19**, 536.
—— (1913). *Arch. Anat. Physiol.* p. 240.
Slowtzoff (1903). *Arch. ges. Physiol.* **95**, 158.
Strasburger (1893). *Hist. Beitr.* **5**, 37.
Terroine (1924, 1). Réunion Pléniaire Soc. Biol. Paris, p. 46 (Masson).
—— (1924, 2). *Compt. Rend. Acad. Sci.* **178**, 1022.
Terroine, Feuerbach and Brenckmann (1923). *Arch. internat. Physiol.* **20**, 466.
—— —— —— (1924). *Arch. internat. Physiol.* **22**, 233.
Terroine and Roche (1925). *Arch. internat. Physiol.* **24**, 356.
Terroine, Trautmann and Schneider (1926). *Ann. Physiol.* **20**, 468.
Truszkowski (1926). *Biochem. J.* **20**, 441.
Warburg (1909). *Z. physiol. Chem.* **66**, 305.
—— (1912). *Arch. ges. Physiol.* **145**, 277.

CXXXVIII. STUDIES IN PURINE METABOLISM.

IV. THE NUCLEAR-PLASMIC RATIO IN DOGS IN CARBOHYDRATE AND PROTEIN FEEDING, AND IN STARVATION.

By RICHARD TRUSZKOWSKI.

From the Biochemical Laboratory, Faculty of Veterinary Medicine, Warsaw University.

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In Part I of this series [1926] it was shown that the nuclear-plasmic ratio of rats is slightly lowered on death from total inanition, whence it was concluded that protein reserves do not exist in the cytoplasm of rats. In this paper similar measurements made on the liver and skeletal muscles of dogs under various conditions of alimentation will be described.

Whilst the theoretical and historical aspects of the question of protein reserve were considered in the above-mentioned paper (Part I), as certain other researches have since come to my notice, I shall commence with their discussion.

Terroine and co-workers have, in a series of papers on inanition, expressed their views on the protein reserve question. Thus Terroine, Feuerbach and Brenckmann [1923] considered that, as the percentage protein content of mice and small birds increases during inanition, protein reserves cannot exist in these animals. In 1926, Terroine, Trautmann and Schneider express the opinion that, if protein reserves do exist, they must be very small in comparison with carbohydrate and fat reserves. Since, however, they found an increase in the total nitrogen content of chicken liver and muscle after protein feeding, they conclude that a limited storage of proteins may exist.

A number of other authors such as Friske [1909] and Junkersdorf [1921] have observed that the nitrogen content of various organs rises in protein feeding as compared to carbohydrate feeding, and have hence drawn the conclusion that this is due to protein storage.

The experiments conducted in this paper show, however, that the premises upon which such arguments are based are misleading, since no analysis is made of the repartition of this nitrogen between nucleus and cytoplasm. This question will be discussed more fully later.

Rubner [1911] describes an experiment of Thomas in which, in a man maintained on a carbohydrate diet after a period of protein feeding, nitrogen elimination falls for nine days, and this Lusk [1924] explains as being due to the gradual solution of "deposit protein," which may amount to 3 % of total

proteins. A very detailed study of nitrogen hunger in man has been published by Smith [1926], whence it appears that, whilst uric acid-nitrogen excretion is practically constant throughout the period of nitrogen hunger, total nitrogen falls constantly during the experimental period of over three weeks; whilst at first sight this research would appear to support the view that protein storage exists, we shall adduce evidence in a later part of this paper that the results of this author might be interpreted otherwise.

Finally, Tichmeneff [1914] and Cahn-Bronner [1914; Berg and Cahn-Bronner, 1914], on the basis of metabolism experiments, conclude that the liver may serve as a storehouse of protein matter.

It is our opinion that metabolism experiments alone, unsupplemented by direct analyses, cannot yield decisive results in such cases. In particular, the question of the fate of retained nitrogen cannot be answered merely by drawing up a nitrogen balance sheet, whilst the evidence on which protein storage in mammals is accepted is extremely slender.

In order to throw further light on the above question, analyses have now been made with the object of determining the total nitrogen, protein and purine-nitrogen contents and the nuclear-plasmic ratio of the liver and skeletal muscles of dogs on a carbohydrate and a protein diet, and in inanition. Should any protein be stored in the cytoplasm of the cells of the above tissues, one would expect the ratio of nuclear to cytoplasmic nitrogen to fall, since we have no reason to believe that reserve protein is a nucleoprotein. Similarly, if in hunger reserve protein is used up, the nuclear-plasmic ratio should rise accordingly.

Experimental.

A series of dogs was fed for three weeks on oatmeal and potatoes—a diet which contains sufficient protein to cover nitrogen catabolism, but which is mainly carbohydrate. The dogs were then killed by injection of chloroform into the heart, the liver and about 100 g. of mixed skeletal muscle being then removed. The liver, after expression of blood and washing, was weighed and minced; from the well mixed mince, 50 g. were taken for hydrolysis and 20 g. were dried to constant weight. In certain cases a further 20 g. portion was extracted with hot alcohol and dried. Portions of the powdered residue were then weighed out, and nitrogen determined by the Kjeldahl method. In this way the percentage of protein nitrogen could be determined, as distinct from total nitrogen, which was determined from the hydrolysate of the 50 g., as described in a previous paper [Truszkowski, 1926]. Purine-nitrogen was determined by the method of Krüger and Schittenhelm [1905] and the nuclear-plasmic ratio was calculated as $N_P/N_T - N_P$.

Skeletal muscle was taken from all four limbs and from various parts of the body; as far as possible fat and sinews were removed, and the remainder minced and treated as for liver, except that protein-nitrogen was not separately determined.

The results obtained are shown in the first parts of Tables I and II. Table I gives the values found for liver. The weight of this organ varies from 151 to 320 g., this depending largely on the weight of the dog examined. Percentage dry substance varies from 20·7 to 25·9, average 24·4. The lowest nitrogen content is 1·898 %, and the highest 2·800 %; for dry weight from 7·33 to 11·46 %. Protein-nitrogen, as determined for dogs 1, 4 and 5 is almost identical with total nitrogen in the first case, and 10 and 5 % higher in the other two cases respectively, indicating an extraction of non-nitrogenous constituents, probably lipins, by the alcohol. Purine-nitrogen varies from 76·4 to 93·3, average 86·7 mg. % live weight and from 325·5 to 396·8, average 355·5 mg. % dry weight. The nuclear-plasmic ratio varies from 32·71 to 47·26—a maximum deviation of about 20 % above and below the average of $40{\cdot}53 \times 10^{-3}$.

Table I. *Liver in carbohydrate and protein feeding, and in starvation.*

No.	Weight of liver g.	% of dry substance	% N live weight	% N dry weight	% Protein-N live weight	% Protein-N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio $\times 10^{-3}$	Nature of feeding
1 ♂	240	25·6	2·055	8·03	1·979	8·097	89·9	350·9	45·69	3 weeks carbohydrate
2 ♀	267	25·9	1·898	7·33	—	—	84·3	325·5	46·46	3 ,, ,,
3 ♂	329	25·4	2·837	11·16	—	—	89·9	353·6	32·71	3 ,, ,,
4 ♂	151	25·2	2·800	11·14	—	12·30	—	—	—	3 ,, ,,
5 ♂	231	20·7	2·372	11·46	—	11·93	76·4	369·2	33·30	3 ,, ,,
6 ♂	320	23·5	2·066	8·79	—	—	93·3	396·8	47·26	3 ,, ,,
Mean	—	24·4	2·335	9·65	—	—	86·7	355·5	40·53	
7 ♀	225	24·3	3·311	13·62	—	—	124·4	511·8	39·02	3 weeks meat
8 ♂	280	29·5	3·334	11·31	—	—	—	—	—	3 ,, ,,
9 ♂	325	22·7	2·847	12·54	—	—	128·3	565·2	47·19	10 ,, ,,
10 ♂	383	26·5	4·489	16·94	3·303	13·62	144·7	546·2	33·33	12 ,, ,, *
11 ♀	840	24·0	2·926	12·19	—	—	114·5	476·9	40·70	12 ,, ,,
Mean	—	25·4	3·341	13·32	—	—	127·5	525·0	39·69	
12 ♂	141	25·2	4·064	16·11	3·929	16·20	167·6	663·7	43·02	14 days inanition
13 ♂	200	24·3	3·181	13·09	—	—	123·8	509·3	40·49	23 ,, ,,
14 ♂	86	26·5	3·931	14·84	—	—	182·9	690·2	48·79	35 ,, ,,
Mean	—	25·35	3·725	14·68	—	—	158·1	621·1	44·10	

* Dog No. 10 was killed by strychnine.

A second series of dogs was fed for periods of from 3 to 12 weeks on boiled horseflesh, and the same values determined as before (Tables I and II, second portions). Here we may note that the weights found for the liver are on the whole greater, although the dogs were about the same size. The percentage dry substance is also slightly greater, total nitrogen is 43 and 38 % higher, and purine nitrogen 47 and 41 % higher for wet and dry weights respectively. As a result the nuclear-plasmic ratio shows a slight increase of from 38·58 to $39{\cdot}69 \times 10^{-3}$. One dog of this series was killed by strychnine (No. 10), and this dog shows exceptionally high values for total nitrogen, although protein-nitrogen gives figures comparable with the total nitrogen found in the other four cases. These high values may be partly due to loss of glycogen from the liver, and partly to the presence of extractive non-protein-nitrogen.

Three dogs were subjected to inanition, water being allowed freely. Two were taken for analysis immediately upon death from hunger, and one was killed whilst in the last stages of exhaustion. The duration of life under these conditions depended upon the room temperature, which was, however, never above 10°, and upon the initial state of nourishment. Here the percentage dry substance in the liver (Table I, Part III) is almost identical with that in meat feeding, total nitrogen is 10 % higher and purine-nitrogen about 20 % higher. Protein-nitrogen, as determined for dog No. 12, is almost identical with total nitrogen. The nuclear-plasmic ratio is about 10 % above that found in carbohydrate and protein feeding, pointing to a greater depletion of non-purine than of purine constituents of the liver.

Table II shows, on the whole, a similar state of affairs in muscle to that in liver, under the same conditions.

Table II. *Skeletal muscle in carbohydrate and protein feeding, and in starvation.*

No.	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio $\times 10^{-3}$	Nature of feeding
1♂	21·1	2·398	11·36	47·1	223·2	20·03	3 weeks carbohydrate
2♀	24·5	2·575	10·51	58·0	236·9	23·07	3 ,, ,,
3♂	21·3	2·742	12·88	58·2	273·6	21·70	3 ,, ,,
4♂	21·8	2·531	11·61	55·1	252·8	22·26	3 ,, ,,
5♂	18·0	2·310	12·83	49·2	273·3	21·76	3 ,, ,,
6♂	20·3	2·527	12·47	57·4	283·4	23·25	3 ,, ,,
Mean	21·2	2·514	11·88	54·2	256·2	22·01	
7♀	26·3	3·181	12·09	72·1	274·0	23·18	3 weeks meat
8♂	22·3	3·087	13·85	84·3	377·9	28·06	3 ,, ,,
9♂	24·8	3·357	13·54	87·7	353·8	26·84	10 ,, ,,
10♂	23·8	3·931	16·52	81·7	343·4	21·23	12 ,, ,,
11♀	24·0	3·480	14·50	77·3	322·1	22·64	12 ,, ,,
Mean	24·2	3·407	14·10	80·6	334·2	24·28	
12♂	18·8	3·174	16·88	—	—	—	14 days hunger
13♂	24·3	3·527	14·52	83·68	344·3	24·30	23 ,, ,,
14♂	19·5	3·249	16·66	65·66	336·7	20·63	35 ,, ,,
Mean	20·8	3·317	16·02	74·67	340·5	23·02	

In meat feeding, dry substance is 14 % higher than in carbohydrate feeding, total nitrogen is 36 and 19 %, and purine-nitrogen 49 and 30·4 % higher for wet and dry weights respectively. Here, too, the nuclear-plasmic ratio is higher by 10 %, greater variations existing between the various values obtained in the meat than in the carbohydrate series, namely, from 20 to 23 in the latter and from 21 to 28 in the former. This difference is due to variable total nitrogen contents in the meat series, from 3·087 to 3·931 %. In hunger, the percentage solid substance falls to slightly below the carbohydrate level, with a corresponding drop in total nitrogen content, as a result of which the latter value for dry muscle is the highest of all obtained, corresponding to practically pure protein. The purine content is slightly lower than in meat feeding, and the nuclear-plasmic ratio lies between the two previously obtained

values, showing that in dog muscle the ratio between cytoplasmic and nuclear nitrogen is constant, in spite of considerable variations in the individual values obtained for total and purine-nitrogen.

Discussion.

The above results show that in dogs the nuclear-plasmic ratio of liver and skeletal muscle is in both cases increased by liberal meat feeding as compared to carbohydrate feeding. Hence it follows that, in these circumstances, not only is no specific protein laid apart in the cytoplasm of the cells of the organs in question, but proportionally more nuclear substance is formed. A further significant difference which may be observed is the considerable rise in the nitrogen content of the muscle and liver in meat feeding, amounting to from 30 to 50 % for both nucleus and cytoplasm. This indicates that nitrogen retention may be due not only to hyperplasia but also to an actual higher concentration of nitrogen in the cells; this difference is, further, too great to be explicable on the basis of different glycogen content. That this is due partly to a diminution in water content is shown by the greater percentage content of dry substance, particularly in muscle. This dehydrating effect of a protein diet has been shown by Bischoff and Voit [1860], and the above results afford, therefore, a confirmation of this early work.

In starvation the water content of skeletal muscle is of the same order as in carbohydrate feeding, whilst the nitrogen and purine contents are only slightly below the meat level. The values found for the nuclear-plasmic ratio are not higher than those found in the previous series, so that here there is no evidence for the existence of protein reserves in muscle cells.

In the case of the liver, dilution cannot be observed in hunger, the mean value found for percentage of dry substance being higher than in carbohydrate feeding. All other values are higher than in the previous series; in particular, high values are found for purine-nitrogen, which is 19 % above meat value, whereas total nitrogen is only 11 % above. In this case, therefore, it would seem that the nucleus has lost proportionally less than the cytoplasm, and consequently that the liver cells may contain a limited store of reserve protein, whose amount cannot be increased by liberal protein feeding, and which is called upon in extreme emergency. On the whole, however, it appears that protein is stored by the body not as a specific deposit protein, but rather by the proliferation of cells, accompanied by greater protoplasmic content of the latter, this difference being over 40 % in the case of the liver, and close on 40 % in the case of skeletal muscle.

Viewed in this light, various arguments advanced in favour of protein storage to a large extent lose their point. Thus the experiments cited in the introduction (Junkersdorf, Friske, Terroine), in which the nitrogen content of muscle and liver rises after protein feeding, are confirmed, but as the increase affects both nucleus and cytoplasm to the same extent, they cannot be considered to afford any evidence whatsoever that a specific protein is laid down.

Nor can the gradual diminution in nitrogen excretion observed during specific nitrogen hunger be put forward as proof of protein storage. It has been shown in this paper that in protein feeding the cells may contain 40 % more nitrogen than in carbohydrate feeding. This 40 % probably corresponds to Rubner's [1911] "improvement quota," and appears to be used up in nitrogen hunger; at the same time nuclear material is, however, catabolised. In nitrogen equilibrium the nitrogen of the entire cell is excreted as urea and uric acid. In nitrogen hunger, on the other hand, amino-acids derived from the autolysis of body tissues are not deaminated but are used for the production of new protein in surviving cells; this re-utilisation of protein material appears to become more and more complete as the body cells approach the carbohydrate diet level of protein content. At the same time, uric acid excretion, which represents nuclear catabolism, should remain constant, since (cf. Part II) purines are synthesised with comparative facility by the mammalian organism, but are not available for resynthesis of nucleic acids if introduced into the blood-stream. The gradual fall in nitrogen elimination during nitrogen hunger, and the constant excretion of uric acid may, therefore, be explained without the necessity of introducing the conception of specific protein storage.

Summary.

1. The chemical nuclear-plasmic ratio of the liver and of skeletal muscle of dogs has been determined, under various conditions of alimentation.

2. The total nitrogen and purine contents of the liver are respectively 43 and 47 % higher in dogs fed with meat than in those fed on carbohydrates.

3. The nuclear-plasmic ratio of liver tissue in meat-fed dogs is almost the same as on a carbohydrate diet, indicating that a liberal protein diet does not lead to the formation of specific deposit protein.

4. The content of solid substance is 14 % higher in skeletal muscle of dogs fed on a meat than on a carbohydrate diet, pointing to a certain dilution of the tissues in the latter case.

5. The total nitrogen and purine contents of skeletal muscle are respectively 35 and 49 % higher in dogs fed with meat than in those fed on carbohydrates.

6. The nuclear-plasmic ratio of skeletal muscle in meat-fed dogs is slightly higher than that encountered in carbohydrate feeding; hence specific reserve protein is not laid down in the muscles as a result of exclusive protein feeding.

7. In starvation, the nuclear-plasmic ratio of the liver is 10 % above normal, pointing to the possible presence of specific reserve protein in this organ.

8. Under similar conditions, the nuclear-plasmic ratio of skeletal muscle is unchanged, indicating that deposit protein is absent from this tissue.

In conclusion, I wish to acknowledge my gratitude to Prof. St. J. Przylecki, who suggested this research and whose constant help and advice were indispensable to its accomplishment.

REFERENCES.

Berg and Cahn-Bronner (1914). *Biochem. Z.* **61**, 434.
Bischoff and Voit (1860). Gesetze der Ernährung des Fleischfressers, p. 211.
Cahn-Bronner (1914). *Biochem. Z.* **66**, 289.
Friske (1909). *Landw. Versuchsstat.* **71**, 441.
Junkersdorf (1921). *Arch. ges. Physiol.* **186**, 254.
Krüger and Schittenhelm (1905). *Z. physiol. Chem.* **45**, 14.
Lusk (1924). Science of Nutrition (Philadelphia), p. 276.
Rubner (1911). *Arch. Anat. Physiol.* pp. 61, 67.
Smith (1926). *J. Biol. Chem.* **68**, 21.
Terroine, Feuerbach and Brenckmann (1923). *Arch. internat. Physiol.* **20**, 466.
Tichmeneff (1914). *Biochem. Z.* **59**, 326.
Truszkowski (1926). *Biochem. J.* **20**, 437.

CXXXIX. REACTION OF FATTY EXTRACTS OF CERTAIN ORGANS WITH THE ANTIMONY TRICHLORIDE TEST FOR "VITAMIN A."

(PRELIMINARY NOTE.)

By WILLIAM H. WILSON.

From the Department of Physiology, Faculty of Medicine, Cairo.

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The test as described by Carr and Price [1926] is thought to indicate the presence of the fat-soluble vitamin A. It is carried out as follows: 0·2 cc. of a 20 % solution in chloroform of the fat to be tested is added to 2 cc. of a 30 % solution of $SbCl_3$ in chloroform. A blue colour results which varies in depth with the amount of the reacting substance present; the colour fades rapidly, the time taken for complete disappearance depending on the depth of the original colour.

It appeared to be of interest to examine the comparative amount of the reacting substance contained in the fatty mixture extracted from the organs, in particular the liver, of man and other mammals without necessarily assuming this to be vitamin A.

Method.

(1) From 100 to 200 g. of liver, or other organ, was passed through a mincing machine into three times the volume of 94 % alcohol, the purpose of the procedure being mainly to facilitate the subsequent drying. The alcoholic extract (holding about 77 % alcohol) was found to contain traces of the reacting substance; the amount lost was however negligible.

(2) After 24 hours, during which time the material was occasionally stirred, the residue was strained off, squeezed out in a cloth and dried in a hot-air oven at about 95°.

(3) The dried material was ground to a fine powder in a mill, the powder being then extracted with ether (at room temperature in a flask) for 24 hours. At the end of this time the residue was filtered off and washed with ether on the filter.

(4) The filtrate was collected in a weighed basin, evaporated until there was no further smell of ether and weighed. It may here be mentioned that the yield thus obtained from different specimens of liver was from 1·3 to 2·7 % of the weight of organ dealt with. The method of extraction by infusion in cold ether with occasional shaking appeared to be sufficiently complete for

the purpose aimed at, which was to compare the amounts of reacting substance present in different fatty extracts.

(5) The fatty extract was dissolved in chloroform to make a 2, 10 or 20 % solution, the concentration being determined by the result of a preliminary test, carried out as described below, to show the comparative richness of the extract in the reacting substance.

The extracts were of a brownish yellow colour of varying depth; those from human liver and other tissues had the consistence of a thick oil but those from ox and sheep's liver were solid at 25°.

The intensity of the reaction with $SbCl_3$ had no relation to the depth of coloration of the extracts.

The liver extracts were tested for the presence of bile pigments with alcoholic iodine, the result being negative. The absence of any change of colour with iodine indicated also that carotene was not present.

In testing the fatty extracts of liver for bile pigment a glass rod wetted with fuming HNO_3 was drawn across a thin layer of the extract on a white porcelain surface; a very deep purple to a bluish pink colour was obtained, fading rapidly to pink and then disappearing. The depth of colour had no relation to the darkness of the brownish yellow coloration of the extracts but was found to be roughly proportional to the intensity of the reaction with $SbCl_3$. The reaction (with HNO_3) was therefore used as a preliminary test to determine the probable richness of different extracts in vitamin A.

To obtain a quantitative expression of the comparative amount of the reacting substance in extracts from different specimens, the blue colour produced with $SbCl_3$ was matched against solutions of indigo-carmine of known concentration. The standard taken was a sample of cod-liver oil which, when tested in 20 % solution, gave a colour matched by a $\frac{1}{3000}$ solution of indigo-carmine. This was given unit value, the content in vitamin A being expressed in multiples or fractions of the unit.

A colorimeter was not used, the comparison being made in test-tubes of the same internal diameter.

It appeared to be of interest to examine the unsaponifiable residue of the liver extracts. Two experiments were done, saponification being effected by boiling with alcoholic potash under a reflux condenser. In the first the saponified material was extracted with ether, the ether evaporated and the residue dissolved in $CHCl_3$; it gave a very strong reaction with both HNO_3 and with $SbCl_3$. In the second 15 g. of the mixed fatty extracts from different specimens of liver were saponified and the material was extracted with light petroleum. This was evaporated, an almost colourless residue being obtained of which 0·02 g. was soluble in $CHCl_3$. Tested in 0·2 % solution this residue had a unit value of 100. It contained cholesterol, the amount of which was not determined. The material thus obtained, although evidently very rich in the reacting substance, gave only a faint reaction with fuming nitric acid. To find some explanation for this rather unexpected result, the saponified

liquid, which had already been extracted with light petroleum, was further extracted with ether, a yellow residue being obtained on evaporation of the ether which gave a strong colour reaction with HNO_3 and a comparatively slight reaction with $SbCl_3$.

In spite therefore of the apparent correspondence of the results of the two tests, when applied to the fatty extracts examined, the reactions appear to be due to the presence of two different substances. Both are found in the unsaponifiable residue, the substance reacting with $SbCl_3$ being however very much more soluble in light petroleum than is the substance giving a purple colour with HNO_3.

The method of colorimetry used would not serve for exact measurements; it gives however a comparative numerical expression of the content in vitamin A of the extracts which is approximately correct.

The subjoined table gives the results of observations on the extracts of human or other origin and of some other materials.

Table I.

Various extracts.

Source of extract	Value in vitamin A	Source of extract	Value in vitamin A
Cod-liver oil A	1	Kidney. Human	Trace
„ B	0·5	Liver. Guinea-pig	3·5
„ C	0·25	„ Ox (*a*)	10
Butter. Foreign	0·05	„ Ox (*b*)	11
„ Local (Cairo) 2 samples	0	„ Water-buffalo	6
Human Milk (Fats) 8	0·05–0·025	„ Camel	12·5
Heart. Sheep	0	„ Sheep (*a*)	7·5
Suprarenal. Ox	0·33	„ Sheep (*b*)	8
Spleen. Human	0	Snail (from 20 glands)	0
Placenta. Human, 2	0		

Extracts of Human Liver.

Source and cause of death	Value in vitamin A	Source and cause of death	Value in vitamin A
1 Child. Bronchopneumonia ...	25	13 Man. Pneumonia	2·5
2 Man. Phthisis	25	14 „ Pneumonia	2
3 Child. Burns	16	15 Child. Bronchitis	2
4 Man. Electric shock ...	12·5	16 Woman. Burns	1·5
5 „ Stenosis of larynx ...	12·5	17 Man. Pneumonia	1
6 „ Heart failure	10	18 „ Nephritis	0·83
7 „ Pneumonia	6	19 „ Aortic disease (syphilitic)	0·4
8 Woman. Burns	5	20 Man. Asphyxia	0·25
9 Child age 3 days. Exposure. Meconium from same ...	0	21 Child. Ill-developed, age 2 months. Corneal ulcers ...	0·2
10 Child (premature) 8 days. Pneumonia	3·5	22 Man. Pellagra	0·1
11 Child. Acute enteritis ...	4·5	Unsaponifiable residue of mixed liver extracts ...	100
12 Man. Heart failure	3·5		

Note. In regard to some of the results recorded in the table it is to be noted that a negative result with Carr's test does not demonstrate the absence of vitamin A, traces which are shown by the $AsCl_3$ test of Rosenheim and Drummond [1925] not being detected with $SbCl_3$.

My colleague Dr A. Hassan has been good enough to carry out some comparative experiments with the two tests which indicate that the former ($AsCl_3$) is between two and three times as sensitive as the latter but that in regard to the determination of the relative richness in vitamin A of different specimens of fatty extracts the two tests agree in their results.

It would be entirely premature to draw any conclusions as to the importance or otherwise of the amount of vitamin A found in the liver in relation to the diseases tabulated. In most of the cases no information was available as to the nature of the diet. The majority of the individuals had either been in hospital for too short a time for the diet supplied to have had any influence or had died outside hospital under conditions which necessitated a medico-legal *post mortem* examination. It may however be of interest to consider very briefly the cases in which there appears to be a great deficiency of vitamin A in the liver extract. These are numbered 20, 21 and 22.

Case 20. This case was that of a man who was buried by a fall of sand when excavating in a sand-pit. There was no evidence of what disease if any he may have suffered from. He belonged to the poorer class and it is unlikely that his diet differed much from that of many of the other cases recorded in whom the liver was found to be rich in vitamin A.

Case 21. The condition of the cornea in this case was suggestive of a deficiency in vitamin A. The child had scarcely grown, although for a month previous to death it had been under treatment in the Children's Institute at Kasr el Aini Hospital. While in hospital the child received partly artificial, partly breast-feeding (a healthy wet-nurse) with only a temporary increase in weight.

Case 22. This was a case of advanced pellagrous insanity, the man having been an inmate of the Khanka Asylum for a long period before his death. I have to thank Dr Miller, the Director of that institution, for kindly supplying the material. The amount of fatty material extracted was 2 % of the weight of liver taken, about the normal quantity.

The great deficiency of vitamin A in the liver extract need not be assumed to have any etiological connection with the disease (pellagra); to establish this the examination of a sufficient number of early cases would be required. It is in fact more probable that this deficiency is related to the fact demonstrated by Roaf and the writer [1918] in an experiment on five comparatively early cases of pellagra that the absorption of fats from an ordinary mixed diet is deficient to the extent of about 19·5 % of the fat intake. In an advanced case, such as No. 22, the extreme atrophy of the mucosa of the small intestine, in particular of the villi, would doubtless exaggerate the defective absorption.

If the substance giving the reaction be regarded as identical with vitamin A[1], the observations on the livers of animals used for food would indicate the value of liver as a foodstuff, especially for children. In the cases recorded in

[1] Apart from the evidence given by Carr and Price [1926] for the identity of the reacting substance with the vitamin, the following facts are in support of the view: (*a*) the results with the $AsCl_3$ and $SbCl_3$ tests give comparable estimates of the amount of the reacting substance present; (*b*) the substance giving the reaction is gradually destroyed (in solution) by exposure to diffused daylight and air at the laboratory temperature; (*c*) the substance is found in the unsaponifiable residue. Wokes and Willimott [1927] in a recent paper on the effect of heat and oxidation on cod-liver oil state that in their observations with different colour tests for vitamin A the $AsCl_3$ and $SbCl_3$ tests were in agreement when used quantitatively.

the table (ox, sheep, camel, water-buffalo) the average percentage of fatty extract was 2·5 %, the average value of which in vitamin A was 9·1 %. On the basis of these figures 100 g. of liver would have as regards vitamin A the same value as 22·5 g. of cod-liver oil.

Summary.

Although in many respects the observations must be regarded as of a preliminary nature, the following conclusions appear to be justified.

1. The fatty extract from the human liver contains a substance giving the same colour reactions as vitamin A in an amount which may be equal to 25 times that found in a good sample of cod-liver oil.

2. The amount of vitamin A found in the liver varies within wide limits and may, under some conditions of disease, be no more than $\frac{1}{250}$ of the maximum amount observed to occur.

3. The substance is present in considerable amount in the liver at birth but is not present, in an amount detectable by the test used, in the placenta.

4. The extract of livers of healthy animals slaughtered for food was found to contain from 6 to 12·5 times the amount of vitamin A present in cod-liver oil.

REFERENCES.

Carr and Price (1926). *Biochem. J.* **20**, 497.

Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.

Wilson and Roaf (1918). Report of a Committee of Enquiry regarding the Prevalence of Pellagra among Turkish Prisoners of War (Egypt), App. VIII, 56–60. See also *J. Roy. Army Med. Corps* (1920), Feb.

Wokes and Willimott (1927). *Biochem. J.* **21**, 419.

CXL. ON THE PIGMENT PRODUCED BY *CHROMOBACTERIUM VIOLACEUM*.

By JOSEPH REILLY and GERALD PYNE.

From the National University of Ireland (University College, Cork).

(*Received July 31st, 1927.*)

The bacterial pigments in general have received very little attention and any systematic investigations in this field have been confined mainly to the pigments produced by pathogenic organisms such as *Bacillus pyocyaneus*. The small amount of pigment formed in most cases is no doubt responsible for this. The extraordinarily intense development of pigment produced in a sample of milk through the growth of *Chromobacterium violaceum*, to which our attention was directed, suggested that this was a pigment which could be produced in sufficiently large quantities for examination, and it was decided to undertake the investigation, particularly as no detailed chemical examination seemed to have been made previously. The accounts of violet bacterial pigments met with in the literature are very fragmentary and somewhat confusing, and deal mainly with their absorption spectra. Lecoq de Boisbaudran [1882] extracted a violet pigment produced by a bacterium, which he does not describe, when grown on starch paste, and records its absorption spectrum. Similar colouring matters obtained from *B. violaceus* and *B. janthinus* are described by Schneider [1895]. The pigment from *B. violaceus* was submitted to a spectroscopic analysis by Hartley [1913], and was considered by him to differ from those described by the authors mentioned inasmuch as it showed more marked absorption in the red. It is possible, however, that the pigment in all these cases was the same—for the earlier spectroscopic observations of Lecoq de Boisbaudran and Schneider were probably not exact enough for the differences to be significant—while the qualitative chemical reactions ascribed to the pigment by all these authors are in fair agreement. The organism producing it is now commonly described as *Chromobacterium violaceum* (Bergey, *Manual of Determinative Bacteriology*). Hartley prepared the pigment by extraction of slices of potato, on which the bacterium grows well, with absolute alcohol, followed by evaporation to dryness. The material obtained in this way can hardly have been very pure. but probably sufficed for the qualitative tests employed and for spectroscopic examination in the absence of any strongly coloured impurities.

For the present work the pigment has been obtained mainly by growth in a lactose broth medium. Isolation and purification presented some

difficulties as the colouring matter is very unstable in alkaline solution in which it dissolves readily, and is not very soluble in organic solvents. An analysis of the material purified as carefully as possible, in the circumstances, gave as a first approximation a formula of $(C_{10}H_{12}O_3N)_n$. Approximately one-fifth of the total nitrogen is removed by the action of nitrous acid, pointing to a value $n = 5$ (or some multiple of five). This was confirmed by a determination of the molecular weight of the substance by a modification of Rast's camphor method devised for the purpose. The value obtained, M = 917, agrees fairly well with that required for $n = 5$.

Preparation and isolation of the pigment.

In the earliest stage of the work sterilised skim-milk was used as a medium for growth as it possessed the advantages of being cheap and of permitting abundant pigment formation. The difficulties met with in filtering this medium and in isolating the pigment in pure form from such a complex mixture led to its eventual replacement by the simpler nutrient lactose broth, made up as follows:

5 g. lactose,
5 g. peptone,
3 g. beef extract,
1 litre of water.

Six litres of the sterile broth contained in a number of flasks were inoculated with a pure culture of *C. violaceum* and kept for about three weeks at a temperature of 20°. The pigment, which is insoluble in water, is found partly in flakes at the bottom of the flasks and partly distributed as very fine particles throughout the medium. It was dissolved out at first by repeated extractions with a mixture of alcohol and ether but this method, though suitable for skim-milk, proved unnecessarily wasteful for lactose broth. In the end it was found simplest to filter off the colouring matter by pouring the culture medium on to large filter papers. Filtration was slow but could not be improved by the use of suction, as the papers became clogged. A stream line filter was also tried but was not successful for the same reason. As soon as all the medium had run through, the residue on the paper was washed with a little water, and allowed to dry in the air. The filter papers were then cut up and extracted with absolute alcohol in a Soxhlet until no more colouring matter appeared to dissolve out. As this process usually took a long time—10 to 12 hours—a "vacuum" Soxhlet was used, to prevent possible decomposition of the pigment by prolonged heating, *i.e.* the extractor was provided with a very long Liebig condenser connected to the water-pump. The temperature of the boiling alcohol in contact with the dissolved pigment could thus be kept below 50° and practically no alcohol was lost. After the removal of as much colouring matter as possible in this way, the filter papers were further extracted by boiling for a few minutes with successive portions of fresh alcohol. These extracts were

filtered, added to the Soxhlet extract, and the whole evaporated to small volume under reduced pressure. From this, the pigment was precipitated by the addition of 3–4 times its bulk of water, filtered under pressure, washed with water, dried on the pump and extracted several times with chloroform to remove traces of fatty substances, etc. The chloroform extract was always coloured greenish-brown, apparently by a second pigment present in small amounts. The residual pigment was now re-dissolved in alcohol, filtered, reprecipitated by addition of water, filtered off, dissolved again in alcohol and concentrated under reduced pressure until only a few cc. remained, and the greater part of the pigment had separated out. The solid was filtered off, dried in a vacuum desiccator and used for analyses and other estimations. A further supply of a presumably less pure material was obtained by the addition of water to the alcoholic filtrate. The total yield of pigment from 6 litres of lactose broth was about 60 mg.

Properties. The solid pigment is a purple-black amorphous substance which decomposes on heating without melting. It is insoluble in water, chloroform and other chlorinated solvents, light petroleum, carbon disulphide and acetic acid. It dissolves in ethyl alcohol to a beautiful bluish-violet solution, in ether (especially ether containing some alcohol), and in acetone to give purple solutions. Although these solutions are strongly coloured, the concentration of dissolved substance for saturation is low—probably not more than about 0·1 %. Pyridine—the best organic solvent observed—contained when saturated at ordinary temperature about 0·5 % of the pigment. Alkalis dissolved the pigment readily to green solutions, which, on heating, rapidly turned blood red, and then slowly passed through reddish brown shades to brown. Some structural change seems to occur on solution in alkali, as addition of acid does not restore the original colour.

Heating a pyridine solution of the pigment with acetic anhydride gave a red solution which was fairly stable to further heating. On the grounds that the pigment might contain a reactive NH_2 group, attempts were made to prepare derivatives by the action of formaldehyde and of toluene-*p*-sulphonyl chloride in pyridine solution respectively. No change was observable although in the latter case boiling was continued for several hours.

A number of tests were carried out on alcoholic solutions of the pigment. These appeared to be fairly stable to reducing agents, remaining unchanged when acted upon by sulphur dioxide, zinc and dilute sulphuric acid, and stannous chloride. Potassium permanganate instantly gave a brownish-yellow solution, while bromine water brought about immediate decoloration.

The action of nitrous acid on the pigment gives a reddish brown solution from which a flocculent brown precipitate, very difficult to filter, eventually settles out. After destruction of the excess of nitrous acid by urea and acidification with dilute sulphuric acid, the brown substance was extracted by *n*-butyl alcohol, washed free from acid with sodium bicarbonate solution, and isolated by removal of the alcohol. The product did not respond to the

Liebermann nitroso-reaction, so that the original pigment does not seem to possess a secondary amine group.

The pigment dissolves in concentrated nitric acid to give an orange solution which becomes more intense in colour on addition of alkali. It does not respond to Millon's test.

Analysis:

Found for pigment	Calc for $(C_{10}H_{12}O_3N)_n$.	Calc. for $C_{50}H_{59}O_{15}N_5$
C = 62·07 %	C = 61·86 %,	C = 61·92 %
C = 6·04 %	H = 6·18 %,	H = 6·09 %
N = 7·14 %	N = 7·22 %,	N = 7·23 %
O = 24·75 % (by diff.)	O = 24·74 %.	O = 24·76 %

From the content of amino-nitrogen and the molecular weight it appears (as will be seen later) that $n = 5$ which would make the formula for the substance $C_{50}H_{60}O_{15}N_5$. The slightly differing formula, $C_{50}H_{59}O_{15}N_5$ is suggested for the pigment as it is in somewhat better agreement with the analytical data.

Action of nitrous acid on the pigment.

An attempt was made to determine the nitrogen liberated from the pigment by the action of nitrous acid in the Van Slyke micro-apparatus. Several difficulties presented themselves. The only solvents in which the pigment was sufficiently soluble to permit the use of amounts even as great as 10 mg. were pyridine and sodium hydroxide solution. The use of the former seemed seriously to affect the method even when a sufficient excess of acetic acid was subsequently introduced to neutralise it, giving low results and much slower evolution of nitrogen when added to a known solution of tyrosine. An alkaline solution of the pigment had therefore to be introduced, although it was almost certain that permanent chemical changes had been produced in the substance by solution in alkali. It was furthermore obvious that if only about one-fifth of the nitrogen could be removed by the action of nitrous acid (as appeared to be the case), the volume of nitrogen obtained from about 10 mg. of pigment would not much exceed 0·2 cc. In these circumstances it was of the utmost importance to see that the blanks were as small as possible. Blank estimations were carried out with a number of samples of sodium nitrite and found, however, to be surprisingly large, in all cases amounting to about 0·5 cc. for an evolution of 25 cc. of nitric oxide. Some values obtained from a sample of Kahlbaum's nitrite were, *e.g.* 0·46, 0·46, 0·59, 0·44, 0·48, 0·45 cc. It was hoped that the gas found in the blanks might be nitrous oxide, or at least contain a definite percentage of nitrous oxide, so that a gas analysis might be used to determine in an actual estimation how much of the total volume was due to the blank and how much to the substance under examination, but an analysis of the "blank" gas in a Bone and Wheeler apparatus proved it to consist entirely of nitrogen. Recrystallisation of the sodium nitrite led to a slightly lower value for the blanks, which were, however, still considerable.

At this stage it was found that Van Slyke [1913] had already remarked on this matter as follows: "The substance in the nitrite which gives the small amount of gas obtained on blank determination we have never been able to identify or remove...." To test the point further we prepared a solution of nitrite by the method recommended by Divers [1899], namely by passing the gases obtained by heating arsenious acid with nitric acid into sodium carbonate solution. The blank from a sample of nitrite prepared in this way was 0·25 cc. for a nitric oxide evolution of 35 cc. In view of these facts it seems possible that part at least of the blank is due to a secondary decomposition of nitrous acid with formation of nitrogen, and consequently cannot be avoided in these determinations. The procedure which had to be adopted finally was to make a determination of amino-nitrogen and follow it immediately by a blank estimation, taking care that the volume of nitric oxide evolved in the two cases should be exactly the same. No great accuracy could be expected for the result in view of the relative proportions of blank and estimation, so that the agreement found for duplicates below is very satisfactory.

(*a*) 8·725 mg. pigment dissolved by warming with 3 cc. *N* NaOH and introduced into the reaction vessel.

Shaken, allowed to stand for a half-hour, and then shaken vigorously for five minutes.

Vol. of moist N = 0·66 cc.; vol. of blank N = 0·46 cc. Temp. 16° Bar. 770 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 19{\cdot}0\ \%.$$

(*b*) 8·255 mg. pigment. Vol. of moist N = 0·352 cc. Blank = 0·17 cc. Temp. 16°. Bar. 767 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 18{\cdot}2\ \%.$$

These values indicate that approximately one-fifth of the total nitrogen is removable by the action of nitrous acid.

Molecular weight. The first attempts to determine the molecular weight of the pigment were made with the Pregl ebullioscopic apparatus, using alcohol as solvent. Although the solution was very deeply coloured, no appreciable rise of boiling point could be detected on addition of the pigment on account of its low solubility and apparently high molecular weight. Acetone, and methyl alcohol, both of which dissolve the pigment to give highly coloured solutions, were also tried as solvents but with as little success. An attempt to use pyridine had to be abandoned on account of the impossibility of obtaining a steady temperature. Fortunately the pigment showed slight solubility in camphor, though only sufficient to give a freezing point depression of about 0·5° by the ordinary Rast method for molecular weight determination. A modification of this method was, however, devised by the authors (and is elsewhere described [1927]), which allowed of the accurate determination of molecular weights from such small depressions of freezing point. The following estimations have been made by its use.

(*a*) 3·77 mg. pigment in 434 mg. camphor. $\Delta = 0{\cdot}38°$.
$M = 923$.

(*b*) 3·14 mg. pigment in 348 mg. camphor. $\Delta = 0{\cdot}40°$.
$M = 910$. Mean value $M = 917$.

$(C_{10}H_{12}O_3N)_4$ requires $M = 776$.
$C_{50}H_{59}O_{15}N_5$ requires $M = 969$.

The molecular weights found point to the latter formula. Although the results are somewhat low, this is not unnatural in view of the very great influence of traces of simpler impurities on the freezing point, and the unavoidable inclusion in the weight of the solvent of camphor on the sides of the containing vessel.

SUMMARY.

The pigment produced by *Chromobacterium violaceum* has been isolated and examined. Determinations have been made of the empirical formula, molecular weight, and nitrogen removable by the action of nitrous acid. The formula $C_{50}H_{59}O_{15}N_5$ is provisionally suggested for the pigment.

The authors desire to express their thanks to M. Grimes, Ph.D., of the Dairy Bacteriology Department of this College, for assistance in the preparation of pure cultures, media, etc., and to the Chemical Society for a grant which partly defrayed the cost of this investigation.

REFERENCES.

Divers (1899). *J. Chem. Soc.* **75**, 85.
Hartley (1913). *Sci. Proc. Roy. Dub. Soc.* **14**, 63.
Lecoq de Boisbaudran (1882). *Compt. Rend. Acad. Sci.* **94**, 562.
Reilly and Pyne (1927). *Sci. Proc. Roy. Dub. Soc.* **18**, 489.
Schneider (1895). Die Bedeutung der Bakterienfarbstoffe für die Unterscheidung der Arten (Karlsruhe).
Van Slyke (1913). *J. Biol. Chem.* **16**, 121.

CXLI. THE PREPARATION OF TAURINE IN CONSIDERABLE QUANTITY.

By WILLIAM OGILVY KERMACK and ROBERT HENRY SLATER.
From the Research Laboratory of the Royal College of Physicians, Edinburgh.

(*Received August 1st, 1927.*)

In order to carry out certain experiments to investigate the action of taurine in its physiological aspects it became necessary to prepare this compound on a considerable scale. Various possibilities of synthesising it were explored but it appeared that in all probability the cheapest and most expeditious method of obtaining the necessary quantity would be to prepare it from some animal product[1], and ox-bile was, under the circumstances, chosen as being most suitable.

Preliminary experiments with fresh ox-bile obtained from the slaughter house, in which the bile was boiled up for eight hours with one-third of its weight of concentrated hydrochloric acid and the product separated from glycine by treatment with alcohol according to the method of Hammarsten [1901] gave yields of 5 g. of taurine per litre of bile. Difficulties were, however, encountered when it was attempted to apply this method on a large scale. For instance it was difficult to obtain three hundred litres of bile from an ordinary slaughter house even over a considerable time, and it was therefore very useful to find that the commercial preparation of sodium tauroglycocholate which can be purchased in large quantities may be used instead of the fresh ox-bile, over which it has many advantages as a source of taurine[2]. It seems, therefore, desirable to put on record the results which have been obtained by the use of this material. Preliminary experiments showed that on a small scale yields of 3–4 g. taurine were obtained from 100 g. sodium tauroglycocholate. On the large scale it was found that 5 kg. of the sodium salt yielded 173 g. taurine. The details of the large scale process follow.

Experimental.

Sodium tauroglycocholate (5 kg.) is dissolved in water (20 l.) and concentrated hydrochloric acid (10 l. $d = 1{\cdot}18$) is added. The mixture is efficiently stirred and boiled under reflux for ten hours. On cooling, the aqueous solution is decanted from the solid hard mass of dyslysin and the latter washed well

[1] Since the work described in this paper was carried out a synthetic method has been described by Marvel, Bailey and Sparberg [1927] which appears capable of giving good yields of taurine from comparatively cheap materials.

[2] It may be noted that in a paper by Rose and Huddleston [1926] in which the effect of taurine on the growth of animals deprived of cystine was investigated references are made to the use of desiccated bile as a source of taurine.

with water. The aqueous solution and washings are evaporated *in vacuo* to about 2 l., and whilst still hot filtered from the sodium chloride (A) which has separated. The filtrate is further concentrated to 300 cc. on the water-bath and again filtered whilst hot (B). The filtrate is diluted with 0·5*N* HCl to 500 cc., poured into absolute alcohol (5 l.), and after standing overnight, the taurine which separates along with some sodium chloride is filtered off, whilst the glycine remains in solution. Recrystallisation of this material from an equal weight of boiling water yields almost pure taurine containing a barely detectable amount of sodium chloride. The compound is washed with absolute alcohol and the alcoholic washings mixed with the filtrate: this precipitates taurine and sodium chloride contained in the mother-liquors. The sodium chloride obtained at the stages A and B (mentioned above) is extracted with a small quantity (about 200 cc.) of boiling water and the solution is filtered hot, the process being repeated if necessary. These filtrates containing taurine, sodium chloride and possibly some glycine are diluted to twice their volume with 0·5*N* hydrochloric acid and the solution is added to ten volumes of absolute alcohol so as to effect separation from the glycine. The precipitated taurine obtained is crystallised from hot water. The mother-liquors from all these crystallisations are evaporated down to a small volume, filtered hot from the sodium chloride which has separated and allowed to cool. The taurine is filtered off and if necessary again crystallised. The combined sodium chloride residues are extracted with boiling alcohol containing potassium hydroxide (2 %) and the hot alkaline extract is neutralised with acetic acid [cf. Abderhalden, 1909]. The comparatively small amount of taurine which separates on cooling is filtered off and purified by recrystallisation from an equal weight of hot water. The total yield of taurine from 5 kg. sodium tauroglycocholate amounts to 173 g.

The alcohol used in the above purification is distilled off and the resulting aqueous solution is evaporated to dryness on the water-bath. The residue is extracted with boiling absolute alcohol, filtered hot, and the filtrate saturated with dry hydrogen chloride. On standing in the ice-chest overnight crystals of glycine ester hydrochloride separate. These are filtered off, washed with a little alcohol, and dried. The yield is 104 g.

The hard mass of dyslysin, which remains after hydrolysing the sodium tauroglycocholate with hydrochloric acid, is not easily attacked by aqueous sodium hydroxide solution but it is soluble in boiling alcoholic potassium hydroxide solution. When the alcohol is removed by distillation in steam an aqueous solution remains from which the bile acids may be precipitated by means of mineral acid. It may be here noted that attempts were made to obtain taurine by alkaline hydrolysis of sodium tauroglycocholate as described by Abderhalden [1909], but on a large scale the considerable quantities of alkali and acid, which must be used, result in the formation of a very large amount of salt and the difficulty of isolating taurine in good yield and in pure condition becomes very great.

The authors desire to express their thanks to Sir James Walker, and the technical staff of the Chemistry Department, Edinburgh University, for assistance in carrying out some of the major operations in connection with this research, and to the Department of Scientific and Industrial Research for a personal grant to one of them (R. H. S.).

REFERENCES.

Abderhalden (1909). Biochem. Arbeitsmethoden, **2**, 665.
Hammarsten (1901). *Z. physiol. Chem.* **32**, 435.
Marvel, Bailey and Sparberg (1927). *J. Amer. Chem. Soc.* **49**, 1833.
Rose and Huddleston (1926). *J. Biol. Chem.* **69**, 599.

CXLII. CARBON MONOXIDE AS A TISSUE POISON.

By JOHN BURDON SANDERSON HALDANE.

From the Biochemical Laboratory, Cambridge.

(*Received August 2nd, 1927.*)

Carbon monoxide is relatively harmless to invertebrates, micro-organisms and plants. Thus Frankland [1888], Linosier [1889] and others, who found that very large amounts inhibited plant and bacterial growth, did not show conclusively that CO is any more poisonous than N_2. J. S. Haldane [1895] showed that cockroaches would live for long periods in a mixture of 80 % CO and 20 % O_2. Warburg [1926] however found that when the proportion of CO to O_2 in a gas mixture was raised to about 5, the O_2 consumption of yeasts and a coccus was diminished. He also showed that strong light neutralised the effect of CO. He concluded that CO combined with a catalyst in the cells, with which O_2 must combine before it can oxidise other substances, and that this catalyst was probably an iron compound analogous to haemoglobin. As the amount of CO needed to cause a given drop in the O_2 consumption is roughly proportional to the partial pressure of O_2, the analogy with haemoglobin can be carried further.

In the experiments here described Warburg's observation is extended to moths, seeds, and rats. Owing to the difficulty of obtaining a standard of respiration the general behaviour of the organism and not its O_2 consumption was observed. Wax moths (*Galleria mellonella*) from a culture kindly put at my disposal by Dr Keilin, were placed in a glass tube through which mixtures containing various amounts of O_2 and CO were passed. The tube containing the moths was kept in a water-bath at 20°, and the gas passed through sampling tubes before and after reaching the animals. Their contents were analysed after each experiment. If the quantities of O_2 differed by as much as 0·2 % the experiment was rejected. The CO content of one or both tubes was also determined by combustion.

In low percentages of O_2 the moths rapidly became sluggish in their movements, fell to the bottom of the tube, and after two or three minutes became immobile even when it was tapped. On readmitting air, even after an hour or more, they recovered completely. In larger but still inadequate amounts of O_2 various degrees of weakness were observed, ranging from inability to fly or climb to immobility save when stimulated. The symptoms were qualitatively the same whether they were due to O_2 want or to a combination of this with CO poisoning. There was little difference of susceptibility between

individuals, but in order to allow for this three moths were always used, and the behaviour of the majority recorded. Such differences occurred in twelve out of the forty-nine experiments on which Fig. 1 is based. Although various degrees of ataxia could be distinguished, behaviour is classified according as the moths were normal, ataxic, or wholly immobile even on stimulation. Six points recording experiments in the absence of CO have been omitted, as have the results of several experiments done under less rigid conditions which were, however, confirmatory.

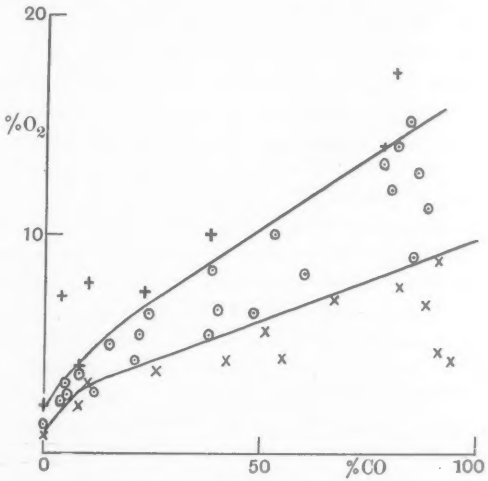

Fig. 1.

+ Moths normal. ⊙ Moths ataxic. × Moths immobile.

In the absence of CO the moths were motionless in mixtures containing less than 1·0 % O_2, normal in over 2·0 %. In presence of 80 % CO about 8·4 % O_2 is needed for mobility and 14 % for normal behaviour, while in intermediate concentrations of CO intermediate amounts of O_2 are required. The lines separating the three areas in the diagram are straight when the CO exceeds 30 % but bend downwards quite definitely at low percentages. The experiments were done in diffuse daylight or electric light, but no definite effect was produced by the proximity of a powerful lamp, probably because the moths are opaque.

The germination of cress (*Lepidium sativum*) seeds in various gas mixtures was next studied. The seeds were placed on wet sand in each of a series of bottles containing known gas mixtures, and their appearance noted daily. Since a preliminary series, which on the whole agreed with later results, showed evidence of leakage in one bottle, the bottles were immersed in a water-bath at room temperature in partial darkness. Two series of twelve bottles each made up in February and March gave quite consistent results. Thus in

the second series in the absence of CO the majority of seeds had split in three days in 8·9 % O_2, in three and a half in 6·9 %, in four in 4·7 %, in eight in 3·0 %, while none split in sixteen days in 2·1 % or 1·4 % though all germinated on admitting air. In the presence of 70 % to 87 % of CO all had split in two and a half days in 19·2 % O_2, in three in 15·3 %, in five in 10·7 %, but after sixteen days only a few had split in 6·2 % and none in 4·1 % or 2·6 %. All germinated on admitting air. By comparing the amounts of germination and growth it was possible to equate the effects of different gas mixtures. It was found that for equally rapid germination and subsequent growth between two and three times as much O_2 was needed in the presence of about 80 % CO as in its absence. No qualitative difference was noted between the effects of CO and of simple O_2 want. When the experiment was repeated in the summer on a smaller scale no change was noted in the susceptibility to O_2 want, but that to CO was somewhat increased, not all the seeds having split after five days in 83 % CO + 11·8 % O_2. This may have been due to the higher temperature or to ageing of the seeds.

The experiments on rats were, of course, complicated by the presence of haemoglobin. Advantage was therefore taken of the fact that mammals can live on O_2 dissolved in their blood under high pressure even when almost all their haemoglobin is combined with CO [Haldane, J. S. 1895]. A small iron chamber was fitted with rubber washers so that its door could be made air-tight, a window for observation, and three taps, two of which were connected with cylinders containing O_2 and CO under pressure; the third could be opened to the outside. When a rat was placed in three atmospheres of O_2 and one of CO its behaviour was almost normal, but in some cases the movements of the hind limbs were rather clumsy. In two atmospheres of O_2 and one or half an atmosphere of CO movement was difficult, but there was little increase of breathing and no convulsions.

There are no exact data as to the affinities of rat's haemoglobin for CO and O_2 at body temperature. At 15° the ratio is about 280 : 1, that for a human haemoglobin being 400 : 1 [Anson, Barcroft, Mirsky and Oinuma, 1924], whereas at 37° the human value is about 250 : 1. If then the temperature coefficient of the equilibrium $HbO_2 + CO \rightleftarrows HbCO + O_2$ is the same in rats and men, the rat's ratio at body temperature is about 175 : 1. Hence at three atmospheres pressure of O_2 and one of CO the rat's haemoglobin was about 98·3 % saturated with CO, the remaining 1·7 % being a very inefficient carrier of O_2 [Haldane, J. S., 1912]. Since a single atmosphere of CO is sufficient to nullify practically all the haemoglobin, the effects of any further addition of that gas can only be on the tissues, since, as will be seen below, the addition of a physiologically inert gas such as N_2 is without effect.

The following experiments, all on the same rat H, are typical.

July 9th

3.50	Pressure in chamber raised to total of 3·1 atmospheres of O_2
3.52	1 atmosphere CO added. Rat at first slightly excited, perhaps by the smell.
4.22	Rat normal. Excess of pressure over atmospheric has fallen from [illegible] per sq. in. to 43. Lower pressure to 15 lb. Raise to 45 lb. with O_2.

July 9th	
4.25–4.38	Pressure repeatedly lowered and then raised with O_2 until the partial pressure of CO was reduced to 0·13 %. The rat was then taken out, and was unaffected.
July 11th	
3.2	Pressure raised to 3·1 atmospheres O_2.
3.4	1 atmosphere CO added.
3.6	2 atmospheres N_2 added.
3.30	Pressure fallen from 77 to 64 lb. Rat normal. Pressure lowered and CO diluted as above.
4.0	Rat removed from chamber, quite normal.
July 13th	
4.55	Pressure raised to 3·1 atmospheres O_2.
4.57	Add 1 atmosphere CO. Rat normal.
5.0	Add second atmosphere CO.
5.1	Deep and rapid breathing.
5.2	Breathing rapid. Rat goes into violent convulsions.

Although the O_2 pressure was subsequently raised to 6 atmospheres the rat's breathing became shallow and it died.

Altogether eight experiments of this type were made. In all cases the addition of sufficient CO caused hyperpnoea and convulsions. If O_2 were at once added and the pressure quickly lowered so as to dilute the CO it was generally possible to save the animal's life. The rats used showed very different susceptibilities to high pressures of CO, all from the same cylinder. Thus while rat H died on exposure to 3 atmospheres of O_2 and 2 of CO, and rat F in 2 of O_2 and 2 of CO, rat B appeared practically normal after four minutes in this mixture, though it died on raising the CO pressure to 3 atmospheres. Finally rat D appeared little affected by four minutes' exposure to 2 atmospheres of O_2 and 3 of CO, though killed by an additional atmosphere of CO. These differences may of course be due to different relative affinities of the catalyst for CO and O_2, but it seems equally plausible to trace them to differences in the cerebral vaso-constriction caused by O_2 [Dautrebande and Haldane, J. S., 1921; Tinel, 1927]. No definite evidence was obtained that poisoning by CO at high pressure can be relieved by high pressures of O_2, but attempts to prove this were rendered difficult by the fact that convulsions due to acute O_2 poisoning set in at about 7 atmospheres pressure of O_2. It is, however, clear that CO has a poisonous action on rats apart from its combination with haemoglobin.

In order to test the possibility that the effects observed were due to impurities in the CO, the gas was made by two different methods. Most of the experiments were made with gas prepared by heating formic and sulphuric acids, and passing the gas evolved through soda or over soda-lime. The analytical figures obtained on combustion suggested that it contained about 2 % H_2. A sample prepared from oxalic and sulphuric acids had indistinguishable physiological properties. The point in Fig. 1 representing a mixture containing 67·1 % CO and 6·99 % O_2 was obtained with this gas. Two of the moths were quite immobile in it, one preserved reflex movements on tapping. Hence, as was to be expected, the mixture was just on the border-line of those producing immobility and ataxia.

All samples of CO had a definite smell generally compared to that of

garlic, but by one colleague to that of tar. This smell was not appreciably diminished by passing the gas over adsorbent charcoal or through bromine water and soda solution, which might be expected to remove large or unsaturated molecules. It is therefore likely that odour is a physiological property of CO [Haldane, J. S., 1921]. It is only observed in fairly high concentrations, which may of course be smelt with safety provided the operation is not repeated too often at short intervals.

Discussion.

The experiments of Keilin [1927] on extracts of yeast, mammalian muscle, oatmeal, and potatoes, make it fairly clear that CO acts by combining with polyphenol or indophenol oxidase, or at least some component of that oxidase system. Since the amount of CO needed to produce a given degree of inhibition depends on the O_2 pressure it follows that both combine with it in the same way. Thus in the case of yeast saccharase, fructose competes with sucrose for the enzyme molecule, and therefore depresses hydrolysis more in weak than in strong sucrose solutions, whereas glycerol and other substances which do not so compete with sucrose depress hydrolysis to a degree nearly independent of the sucrose concentration [Michaelis and Pechstein, 1914]. In this respect CO is clearly comparable with fructose rather than glycerol. In view of the results of Keilin and the great speed with which O_2 must diffuse within a cell it is very unlikely that the substance sensitive to CO is a mere carrier of molecular O_2 like haemoglobin.

If CO combines with a catalyst X normally concerned in oxygen activation, the following reactions may be supposed to occur:

$$X + O_2 \rightleftarrows XO_2$$
$$XO_2 + 2A \rightarrow X + 2AO$$
$$X + CO \rightleftarrows XCO$$

where A is an oxygen acceptor, possibly 2 H atoms from an activated molecule. Let x be the concentration of CO, y that of O_2, $e - p - q$ that of X, p that of XO_2, q that of XCO and a that of acceptor. Let k_1, k_2 be the velocity constants of the first reaction, k_3 of the second, k_4, k_5 of the third. Then when the reactions are proceeding steadily or in equilibrium, as the case may be, we have [Briggs and Haldane, J. B. S., 1925]

$$k_1 (e - p - q)\, y = (k_2 + k_3 a^2)p, \quad k_4 (e - p - q)\, x = k_5 q.$$

Eliminating q we find

$$p = \frac{e}{1 + \left(\frac{k_2 + k_3 a^2}{k_1 y}\right)\left(1 + \frac{k_4 x}{k_5}\right)}.$$

Hence the velocity of oxidation,

$$v = k_3 a^2 p = \frac{k_3 a^2 e}{1 + \left(\frac{k_2 + k_3 a^2}{k_1 y}\right)\left(1 + \frac{k_4 x}{k_5}\right)}.$$

If V be the velocity when the O_2 concentration is so high that v no longer depends on it, and if

$$K_{O_2} = \frac{k_2 + k_3 a^2}{k_1},$$

i.e. the Michaelis constant of the oxygen activator,

$$K_{CO} = \frac{k_5}{k_4},$$

i.e. the equilibrium constant of the third reaction,

$$\therefore v = \frac{Vy}{y + K_{O_2}\left(1 + \frac{x}{K_{CO}}\right)}. \quad \text{.....(1)}$$

$$\left.\begin{aligned} \therefore \left(\frac{V}{v} - 1\right) y &= K_{O_2} + \frac{K_{O_2}}{K_{CO}}\, x, \\ \text{or,} \quad x + K_{CO} &= \frac{K_{CO}}{K_{O_2}}\left(\frac{V}{v} - 1\right) y. \end{aligned}\right\} \quad \text{.....(2)}$$

Now if we suppose that a given degree of asphyxia as measured by behaviour or growth corresponds to a particular degree of reduction in the velocity of oxidation in certain cells, equation (2) should give the relation between the partial pressures of CO and O_2 required to produce it. For with a given degree of asphyxia a and hence K_{O_2} are presumably constant, though they may increase with increasing asphyxia.

Warburg showed that equation (1) did not hold for yeast, but that the initial effect of CO in diminishing oxidation was relatively less than the effect of larger amounts. This might have been due, among other things, to the existence of a component of the total oxidation not affected by CO, or to an increase with increasing asphyxia in the value of a, *i.e.* the amount of oxygen acceptors. Neither of these causes however would cause a deviation from the linear equation (2) such as was found in the case of *Galleria*. The relation however is nearly linear for large values of x, the equations for the two linear parts being $x + 25{\cdot}0 = 6{\cdot}9y$ and $x + 28{\cdot}9 = 13{\cdot}2y$. The agreement in the two values, 25 % and 29 % for the value of K_{CO} is as good as could be desired. Hence the catalyst behaves as if it were half saturated with CO in the absence of O_2 at a pressure of about 27 % of an atmosphere.

The deviation from linearity in low concentrations of CO may be due to several causes. In the first place, the dissociation curves of XO_2 and XCO may deviate from rectangular hyperbolae as do those of the corresponding haemoglobin derivatives. But since the presence of small amounts of CO in a gas mixture containing only enough O_2 partially to saturate the haemoglobin probably facilitates the formation of oxyhaemoglobin [Douglas, Haldane, J. S., and Haldane, J. B. S., 1911] such deviations from the law of mass action as might account for this effect would be in the opposite direction to those of haemoglobin.

It seems at least equally probable that the cells of the moth contain several catalysts of the type considered, having different affinities for CO and O_2, only one however being the limiting factor to oxidation above a certain partial pressure of CO. This possibility is borne out by the very

different effects of CO on moths and cress. In 80 % CO the moth needs 7 or 8 times as much O_2 as when the O_2 is diluted with N_2, the seed only about $2\frac{1}{2}$ times. It is very doubtful whether the difference of about 8° in the temperature will account for so large a difference, which is probably analogous to that between different haemoglobins. If the catalyst differs markedly in different cells of the same organism it is likely that measurements of O_2 consumption would yield a less simple result than qualitative experiments depending on a relatively small number of cells in the nervous system.

The actual O_2 pressures in the tissues during these experiments are of course unknown. Those of CO must be the same as in the gas mixture. As however for a given degree of asphyxia the rate of oxidation in the tissues is presumably constant, the O_2 pressures in the gas mixture must, if the O_2 reaches the tissues by diffusion, exceed that in the tissues by a constant amount, which must be less than 2 % of an atmosphere for the barely ataxic moths, less than 1 % for the barely immobile. The effect of allowing for this difference would be to lower the two curves in the figure by a small amount, constant for each curve. Calculations based on their upper portions would barely be affected, nor would such a correction account for their curvature.

The experiments on rats show that a CO-sensitive substance exists in the mammalian brain, as well as in the muscles where Keilin found it. Presumably it is of the same nature as the muscle catalyst, and hence as that in invertebrates and plants. If so CO, like cyanide, stimulates the respiratory centres by inhibiting oxidation there. The possibility that they are made sensitive to oxygen by a substance such as haemoglobin which changes its properties with the partial pressure of O_2 is rendered very unlikely.

The nature of the catalyst is of course unknown, though it may well be an iron-porphyrin derivative. Keilin's results show that it is a component of the polyphenol oxidase system, but it does not of course follow that the oxidation of polyphenols or related substances is a stage in every oxidative process inhibited by CO. Oxygen activated by the catalyst in question may be required for the oxidation of a variety of substances. As such catalysts have now been found in bacteria, yeasts, higher plants, insects and mammals, they are presumably present in the majority, if not all, of aerobic organisms.

Summary.

1. The movements of a moth and the germination of cress seed are inhibited by CO. The greater the partial pressure of O_2, the more CO is required.

2. Rats living on O_2 dissolved in their blood in presence of sufficient CO to combine with almost all their haemoglobin are killed by the addition of more CO, which must affect some substance in their tissues.

3. It is concluded that cells contain a catalyst of oxidation which is poisoned by CO. Its affinity for CO is determined in the case of the moth. Its affinities differ in different species, and perhaps in different tissues.

REFERENCES.

Anson, Barcroft, Mirsky and Oinuma (1924). *Proc. Roy. Soc. Lond.* B, **97**, 61.
Briggs and Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Dautrebande and Haldane, J. S. (1921). *J. Physiol.* **55**, 296.
Douglas, Haldane, J. S. and Haldane, J. B. S. (1911). *J. Physiol.* **44**, 275.
Frankland (1888). *Proc. Roy. Soc. Lond.* **45**, 292.
Haldane, J. S. (1895). *J. Physiol.* **18**, 201.
—— (1912). *J. Physiol.* **55**, 206.
—— (1921). Respiration.
Keilin (1927). *Nature*, **119**, 670.
Linosier (1889). *Compt. Rend. Soc. Biol.* **41**, 1.
Michaelis and Pechstein (1914). *Biochem. Z.* **60**, 79.
Tinel (1927). *Compt. Rend. Soc. Biol.* **96**, 665.
Warburg (1926). *Biochem. Z.* **177**, 471.

CXLIII. GROWTH AND REPRODUCTION ON SYNTHETIC DIETS. II.

By GLADYS ANNIE HARTWELL.

From the Physiological Laboratory, Household and Social Science Dept., King's College for Women, Kensington, London, W. 8.

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In an earlier paper [Hartwell, 1926] a description was given of various synthetic diets which produced good but not maximal growth in young rats. The importance of a really good synthetic diet for use as a standard in nutrition experiments is self evident, and certain modifications of the foods used previously [Hartwell, 1926] have yielded satisfactory results, although, as yet, no diet has been found which will produce growth equal in rate to that obtained on kitchen scraps. The improvement in reproduction and lactation on these modified diets is even more marked than with growth, and therefore it is thought advisable to publish these results briefly in that they may be of use to others working on similar lines.

The difficulty of obtaining normal reproduction on synthetic diets is commented on by many researchers, while the actual rearing of the young when the mothers are living on such foods is almost impossible. Nelson, Jones, Heller, Parks and Fulmer [1926] write that "one thing is certain, that reproduction is not normal on certain synthetic diets and diets involving restricted food materials." They find that the number of young born may be comparatively few and that the majority die before weaning, although they have carried their experiments through several generations.

Using a basal synthetic diet of caseinogen, salt mixture, butter fat and dextrin, with wheat, rye, barley or corn as sources of vitamin B, Guest, Nelson, Parks and Fulmer [1926] found that rats reproduced normally, but that there was a high mortality among the young, due in their opinion to failure of milk secretion. Hogan and Harshaw [1924] reared four generations on a synthetic diet, but found that the number of litters was subnormal, the mortality of the young high and that any young which survived grew very slowly during the suckling period. Anderegg [1924] found a high mortality among the young even when the protein was given as whole milk powder, and Murphy and Jones [1926] state that on a diet of bran, salts, crisco and starch the rats produced offspring, but had little success in rearing them. Kennedy and Palmer [1926] used crisco instead of lard in the basal diet shown by Evans and Bishop [1923, 1] to be inadequate for reproduction, and

obtained litters in three successive generations of rats. They found, however, that the mother rats had great difficulty in rearing their young even when the diet of each rat was supplemented by 15 cc. of whole milk throughout the lactation period.

Thus the failure to rear their sucklings when the mother is fed entirely on a synthetic diet appears to be universally recognised.

Experimental.

The preparation of the food, weighing the animals, etc., has been described previously [Hartwell, 1926]. The composition of the diets is given in the following table.

	Diets				
	A	*E*	*CE*	*CL*	*CLO*
Caseinogen	20 g.	16 g.	8 g.	14 g.	20 g.
Egg-albumin	—	—	8 g.	—	—
Lactalbumin	—	—	—	6 g.	—
Potato starch	64 g.	64 g.	64 g.	60 g.	64 g.
Butter	12 g.	16 g.	16 g.	16 g.	—
Cod-liver oil	—	4 cc.*	4 cc.†	4 cc.	14 cc.
Salt mixture (Hartwell)	4 g.	4 g.	4 g.	4 g.	4 g.
Marmite‡	5 g.	5 g.	5 g.	5 g.	5 g.
Water	300 cc.	300 cc.	300 cc.	300 cc.	300 cc.

* After first six weeks of experiment.

† After first four weeks of experiment.

‡ During lactation 10 g. of marmite were used instead of 5.

Three generations of rats were reared on diet *A*, but slight alterations in the fat were made from time to time. It was noticed that a few of the young rats developed slightly sore eyes at about 17–18 days of age and as this was first seen in the winter it was thought advisable to increase the butter to 16 g. and decrease the starch to 60 g., thus keeping the percentage composition of the other dietary constituents constant. The increase in butter caused no improvement in the eye condition of the suckling young and therefore 4 cc. of cod-liver oil were added to the diet. This alteration, apparently, led to disastrous results in that the mother frequently died when the young were about to be born (this is described later in the paper) and so the cod-liver oil was omitted from the diet. The duration of the experiment and the alterations in diet are given below.

	Duration of experiment	Alterations in diet
1st generation	June 15, 1926–April 5, 1927	4 cc. cod-liver oil added to diet from March 5, 1927
2nd generation, from first litters	Sept. 9, 1926–March 17, 1927	Butter increased to 16 g. on Feb. 3, and 4 cc. cod-liver oil added March 4
2nd generation, from second litters	Oct. 12, 1926–March 16, 1927	Butter increased to 16 g. on Feb. 3, and 4 cc. cod-liver oil added March 4
3rd generation	Feb. 3, 1927–July 15, 1927	Expt. started with 16 g. butter, March 4 4 cc. cod-liver oil were added; May 4 cod-liver oil discontinued and butter reduced to 12 g.

Results and Discussion.

All the rats fed on the synthetic diets described in this paper were in good condition throughout the experiments. The animals were firm to feel and their muscles were fit; there were no signs of general flabbiness, and except that the rats were slightly smaller at any given age, they could not be distinguished from stock animals fed on kitchen scraps supplemented by bread and milk.

Growth.

The rate of growth on diets *A*, *E*, *CE*, and *CL* was good, though not equal to that of stock animals fed on the kitchen scrap mixture (see Fig. 1). Diets *E*, *CE*, and *CL* produced slightly quicker growth than diet *A*. It is impossible

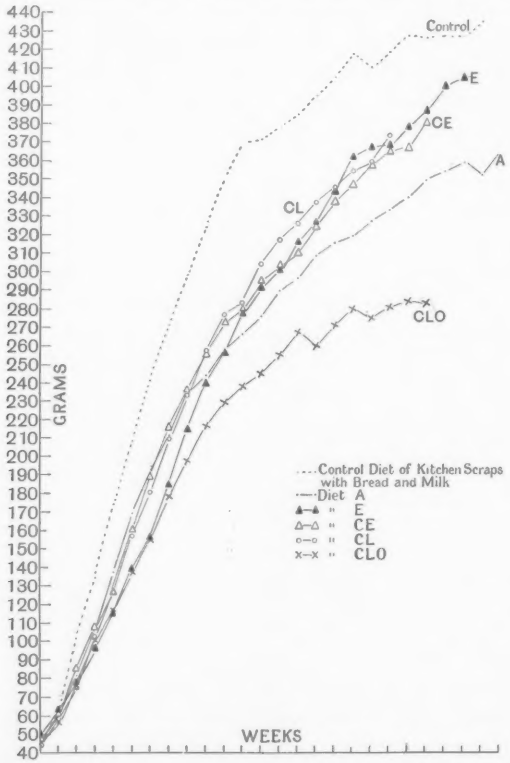

Fig. 1. Growth curves of male rats. Each curve is taken from the average of six animals.

to say what was the cause of this improvement since several factors were varied in each diet. It seems unlikely that the differences in the protein ration are of primary importance here, because diet *E* containing only 16 % of caseinogen produced better growth than diet *A* with 20 % of this protein,

and diets *CE* and *CL* containing two proteins at the level of 20 % produced no better growth than diet *E* with 16 % of one protein. The main difference between diets *E*, *CE*, and *CL* on the one hand and diet *A* on the other is the increased fat content of the former diets, diet *A* containing 12 % of butter, while diets *E*, *CE* and *CL* had not only an increase in butter to 16 % but also 4 cc. of cod-liver oil as well. This involves not only an increase of fat, but also of vitamins A and D in the diet, and possibly vitamin E.

Diet *CLO* gave comparatively poor results for growth, and the curve flattened early as seen in Fig. 1; the rats, however, were in good condition throughout the experiment. Diets *A* and *CLO* are similar in all respects except the fat, the former containing 12 % of butter and the latter 14 cc. of cod-liver oil in place of the 12 g. of butter. It is extraordinary that diet *CLO* produced such poor growth for it *must* have been adequate as regards vitamins A and D, and 5 g. of marmite were used while 2 g. have been found to produce good growth in young rats. It may be that there is an excess of vitamins A and D in this diet and that the poorer growth is due to upsetting the balance of the dietary constituents, or that vitamin E, stated by Evans and Bishop [1923, 1, 2] and Sure [1927] to be deficient, or present in only small quantities, in cod-liver oil, is essential for growth as well as for reproduction. If vitamin E can be stored in the animal body as can vitamin A, the good growth produced in the first few weeks of the experiment and the early flattening of the curve are easily understood.

Reproduction.

(*a*) *Diet CLO.* The fat in this diet was cod-liver oil, which it was hoped to use in place of butter in synthetic diets, in order to have a constant supply of vitamins A and D throughout the experiments, it having been shown by Luce [1924] that the antirachitic value of cow's milk varies with the season of the year. The six does in this experiment produced no litters, though the feeding was continued for 21 weeks, during which time other rats on synthetic diets would each have had two or three families. *Post mortem* examinations showed abnormal uteri and mammary glands, the histological details of which will be published later. The males varied, some appearing almost normal, while others had testes of very low weight. When it was found that this variation occurred, two males with apparently well formed testes were kept for a further period and mated with stock does which had previously reared litters, but no families were born. This experiment appears to confirm the work of Evans and Bishop [1923, 1] that cod-liver oil does not contain vitamin E, regarded by these workers as essential for reproduction. It should however be pointed out that the methods of investigation are not similar; Evans and Bishop used what might be termed a "curative method," while the experiments described in this paper are of the "preventive" type. The assumed absence of a special reproductive dietary factor is a very obvious explanation of these results, but it must be recognised that in both series of experiments the diets contained

large amounts of cod-liver oil and therefore it is possible that an upset of the balance of the various dietary constituents may be a factor of importance. Evans and Bishop [1923, 1] used as much as 24 % of cod-liver oil in one experiment, and they considered that one sample of their oil was at least eleven times as rich in vitamin A as the milk fat of their standard diet.

(*b*) *Diet E.* All six does became pregnant at the normal time, but no young were reared. One doe was killed because of a cancerous growth; three does had first litters either born dead, or the young were eaten immediately after birth, and all three died with their second litters; the other two does died with their first litters. The does appeared perfectly fit and well throughout gestation, but they became comatose and eventually died with the young unborn. Before death they frequently had brief convulsions at intervals during the comatose period. The mothers apparently died from internal haemorrhage; their paws and tongue were quite white, their muscles very anaemic and the liver appeared bloodless. The young seemed normal in all respects. Some of the animals were killed while in the comatose condition, so that the *post mortem* observations should be of more value. As a rule the rats died about two days after the young were due to be born. (This statement is based on general observation of pregnant does; over 2700 litters have been born during the author's research, and by experience it is easy to tell when to expect the birth of the young.) When the six does all died, five other stock females, which had previously reared litters, were put with the males. Four of these had litters, born normally, and three of them reared some of their young.

(*c*) *Diet CE.* This diet gave better results in that some litters were reared, but three out of the six does died, showing the same symptoms as described under diet *E*.

Details of the breeding are as follows:

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
—	5 dead			
2624	7 + 2 dead	5·3	4	31·5
2679	8	6·25	5	30·4
Rat 2				
—	Born dead, rat died the next day			
Rat 3				
—	5. Eaten second day			
—	Born dead			
Rat 4				
—	Died at birth of first litter			
Rat 5				
2589	3. Dead second day.			
2642	1 + 1 dead	4. Eaten third day		
2680	4 + 3 dead	6	4	28·75
Rat 6				
2590	7	6·3	5	30·6
—	Died when litter born			

(*d*) *Diet CL.* The results of this experiment are very similar to those obtained with diet *CE*, three does dying at, or soon after, the birth of a litter.

The few litters born to animals fed on diets *CE* and *CL* compared with those born to rats on diet *A* must not be taken as evidence that the diets *CE* and *CL* are less good for reproduction because these two experiments were of much shorter duration, each being discontinued after a third doe had died.

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
2643	8+2 dead	4·6	3	23
2702	9	6	5	42
Rat 2				
—	Died with first litter			
Rat 3				
2635	2+1 dead	5. Eaten sixth day		
2677	4+2 dead	4·75	2	23·5
Rat 4				
2673	8	5·5	4	29·75
Rat 5				
2638	8	5	5	29·2
—	Died at birth of litter			
Rat 6				
—	Born dead, rat died a few days after			

(*e*) *Diet A*. This diet was by far the most satisfactory for reproduction and three generations were successfully reared. The rate of growth of the second and third generations was slightly slower than that of the first (see Figs. 2 and 3), but all the rats were in good health throughout the experiment and no dietetic disturbances, such as the hair falling out, etc., were ever seen. The slower growth of the second generation is in agreement with the results of Evans and Bishop [1923, 1]. The does in all three generations were fertile and litters were produced as frequently as in animals fed on a mixed diet of kitchen scraps. Two cages of second generation animals were kept, one from first litters and one from second; it was thought possible that if the diet were lacking in any way the second litters might show some effects not found in the first litters, which might have had the advantage of any reserve stores of the mother. However, no such differences appeared, and the two cages of rats were similar in all respects. The rearing of the young was by no means up to standard, although those which survived were usually fit and healthy, but below average weight at weaning. The first generation does reared 85 young out of a possible 156 (all litters were reduced to six on the day after birth); the second generation does from first litters reared 51 out of 100, the second generation from second litters brought up 30 out of 57 and the third generation reared 25 out of 52. This is a great improvement on the results obtained with synthetic diet 1 [Hartwell, 1926] on which only 10 young were reared out of 59. The number of young born to the does on synthetic diet *A* was not up to the controls, 10 being the largest litter, while stock animals produce as many as 18. There was little difference between the numbers born to the first and second generation rats, but there was a considerable drop in numbers per litter of the third generation families.

Details of the reproduction in the first generation are given in the following table:

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
2348	6+3 dead	6	3	22·3
2388	6	5·7	3	30
2442	8	5·7	5	31·4
2483	3	5·6	2	30·5
2527	7	6	6	29·7
—	5. Born dead			
Rat 2				
2347	5	6. Eaten 15th day		
2383	5	5	2	14·5
2432	4	5	4	25·8
2472	1+2 dead	6. Eaten 5th day		
2498	3	5	3	24
2561	2	5·5. Eaten 6th day		
Rat 3				
2352	2	4. Eaten 5th day		
2382	7	5·7	5	28
2437	7	5·7	5	27·6
2475	5	4·2. Eaten 4th day		
2566	4	4·3. Eaten 6th day		
2587	4	4·5. Eaten 19th day		
Rat 4				
2345	3	4·6. Eaten 4th day		
2377	10	5·5	5	35
2406	7	6	6	29·3
2453	4	6·3	4	34
2500	7	5·4	6	29·3
Rat 5				
2346	5	5·6. Eaten 14th day		
2381	9	5·4	5	26·6
2430	4	5	1	38
2470	4	5·3	2	26·5
2514	3. Eaten 2nd day			
2555	1	6. Eaten 5th day		
2580	5	6	5	28·4
Rat 6				
2351	7	5·2	3	29·3
2396	4	6. Eaten 6th day		
2426	7	6·2	6	27·8
2469	4	5·5. Eaten 8th day		
—	Eaten at birth			
2524	6	6	4	30·8
2579	3	5·3	2	21·5

The results of breeding in the second and third generations are not given in detail, but the main points are shown in this table:

	Duration of expt. in weeks	Number of does	Number of litters born	Weight of young on second day (g.) Lowest	Weight of young on second day (g.) Highest	Average weight of young at weaning (g.) (21 days)
1st generation	63	6	37	4·2	6·5	28·4
2nd generation						
From first litters	27	9	28	4·6	6·3	29·1
From second litters	23	7	15	4·7	6·3	26·9
3rd generation	23	8*	15	4·7	6·3	32·5

* Two of these does died at the birth of their first litters.

It is interesting to note that at 21 days the average weight of 87 young from the first generation is 28·4 g., while the corresponding figure from 81 young from the second generation is 28·3 g.

When an experiment was finished a *post mortem* examination was made on each parent animal, and from this examination, all organs appeared to be in a normal condition. This is especially interesting in connection with the third generation rats, where evidence of any dietary deficiencies might have been more marked. The third generation animals had very thick and strong abdominal muscles, the difference being as marked as in the wild rabbit compared with the hutch animal.

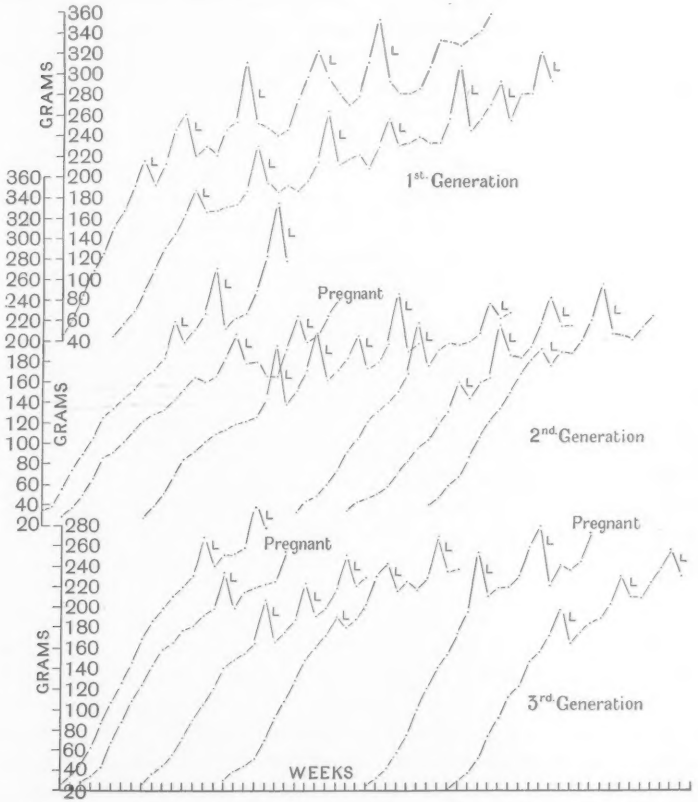

Fig. 2. Growth curves of some of the female rats fed on diet *A*. *L*=litter born.

One of the most striking features of these experiments is the death of the mother rat coincident with the failure of the young to be born. This appears to have a direct connection with the alteration of the fat content of the diet. With diet *A*, the first generation rats were receiving 12 g. of butter and each

doe had 5, 6 or 7 litters, all born normally. In the second generation the fat was altered, but near the end of the experiment, and no catastrophes occurred. The third generation were started from weaning on the increased fat, *i.e.* 16 g. of butter and 4 cc. of cod-liver oil and two does died with their first litters. Consequently the fat ration was immediately changed to 12 g. butter and no more does died. With diets *E*, *CE* and *CL* the fat consisted of 16 g. of butter and 4 cc. cod-liver oil, and 12 of the 18 does died. An experiment is now in progress with a diet similar in composition to diet *A* but with 20 g. butter and no cod-liver oil, to see if the oil is the primary factor or if the actual amount of fat is also of importance. It may be significant that with the fat ration entirely cod-liver oil, no reproduction occurs and that the addition of 4 cc. of the oil to the diet results in a high mortality of the does. It is possible

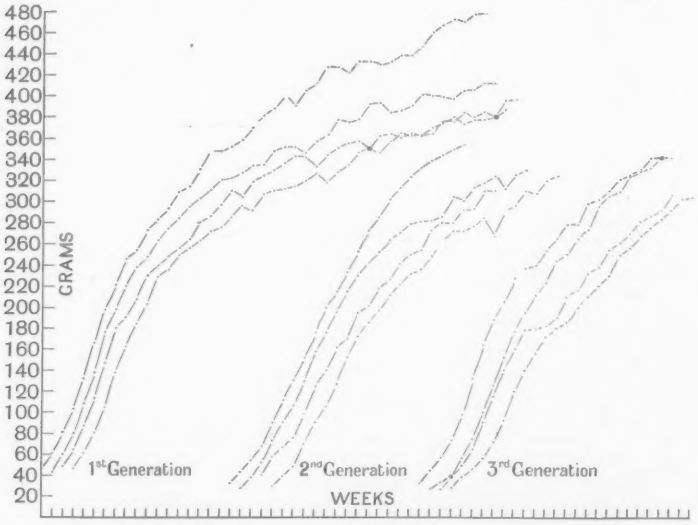

Fig. 3. Growth curves of some of the male rats fed on diet *A*.

that vitamin E is as essential for normal birth as for effective placentation [Evans and Bishop, 1923, 1]. If the cod-liver oil has some inactivating action on the butter, similar to that suggested by Fridericia [1924] for certain oils, it might conceivably diminish the vitamin E content of the diet. Although this is a possible explanation of the results described in this paper, it is merely theoretical at the moment and the problem obviously awaits further elucidation. The evil effects consequent on the addition of cod-liver oil to the diet may be due to some upset of balance of the dietary constituents.

The cod-liver oil was added in the hopes of preventing the sore eyes noticed in the suckling young, but no such improvement was obtained. More recently a similar condition has been found in suckling rats belonging to stock does

fed on synthetic diets during lactation only. These mothers obviously cannot lack vitamin A as they have large reserves of body fat, so it is probable that the eye trouble is the "salt ophthalmia" described by McCollum, Simmonds and Becker [1922; 1925], although the conditions are not absolutely comparable, as these observers were feeding young rats on the diet, while in my experiments the eye trouble developed in the suckling young and was therefore caused indirectly by poor feeding of the mother rat. The soreness was observed as soon as the eyes opened (usually late on synthetic diets compared with stock animals). In some litters no bad eyes were seen, while in others two or three of the young showed the symptoms; in no case was the whole of the litter affected. In several experiments the eye trouble was noticed at the 17th day, and if the young ate for themselves on that day or the next, there was often complete recovery by weaning, *i.e.* the 21st day. This suggests that the diet was deficient for lactation though adequate for growth; it also emphasises the different needs of the rat during lactation and growth, because the young recovered when eating the diet for themselves.

It is possible that lactation throws an even greater strain on the mother than does gestation. In the rat, even if the litter be reduced to six, the combined weight of the young at weaning is sometimes equal to that of the mother, or in the case of a young animal, even greater, while the weight of a new-born litter is small compared with that of the mother, and yet gestation lasts for 23 and lactation 21 days. Thus a litter of six rats at 21 days of age would weigh from 240–300 g., while a new-born litter would rarely weigh over 70–75 g. It seems probable therefore that in a diet suitable for lactation, the carbohydrate should be decreased and other dietary factors increased as compared with the needs for growth.

Diet *A* is probably a good basis, which with some slight modifications, would make a suitable standard for nutrition work. This diet provides for good growth and reproduction. It is not satisfactory for lactation, but is fairly good for the gestation period as the young at birth were either of normal weight or not far below. The fact that the second and third generation animals grew rather less well than the first suggests that diet *A* is not complete, but as three generations were reared on the food it seems probable that all the necessary constituents are present, but that the balance of the various dietary factors needs adjustment. It is possible that a slight increase in mineral salts and perhaps vitamins as well (with a corresponding decrease of starch) is required during gestation, and a larger though similar increase for lactation. Only a few families were reared on diets *CE* and *CL*, but there is some evidence that this mixture of proteins gives better results during lactation, though for growth there seems to be no advantage in supplementing the caseinogen by other proteins. In fact diet *E* with only 16 % of caseinogen produced as good growth as diets *CE* and *CL* which contained 20 % of protein.

SUMMARY.

1. Three generations of rats have been reared on a synthetic diet of butter, caseinogen, potato starch, salt mixture, marmite and distilled water.

2. The use of cod-liver oil instead of butter in this synthetic diet produces less good growth and causes sterility.

3. Synthetic diets are described which produce good growth in young rats, but cause inability of the young to be born and consequent death of the doe.

4. Further evidence is given that the dietary requirements of the rat vary at different phases of its existence.

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REFERENCES.

Anderegg (1924). *J. Biol. Chem.* **59**, 587.
Evans and Bishop (1923, 1). *J. Metab. Res.* **3**, 233
—— —— (1923, 2). *Amer. J. Physiol.* **63**, 396.
Fridericia (1924). *J. Biol. Chem.* **62**, 471.
Guest, Nelson, Parks and Fulmer (1926). *Amer. J. Physiol.* **76**, 337.
Hartwell (1926). *Biochem. J.* **20**, 1273.
Hogan and Harshaw (1924). *J. Metab. Res.* **5**, 111.
Kennedy and Palmer (1926). *Amer. J. Physiol.* **76**, 316.
Luce (1924). *Biochem. J.* **18**, 716, 1279.
McCollum, Simmonds and Becker (1922). *J. Biol. Chem.* **53**, 313.
—— —— —— (1925). *J. Biol. Chem.* **64**, 161.
Murphy and Jones (1926). *J. Biol. Chem.* **69**, 83.
Nelson, Jones, Heller, Parks and Fulmer (1926). *Amer. J. Physiol.* **76**, 323.
Sure (1927). *J. Biol. Chem.* **74**, 45.

CXLIV. THE PHOTOSYNTHESIS OF UREA FROM AMMONIUM CARBONATE.

By WILLIAM ROBERT FEARON and CHARLES BEUNO M'KENNA.

From the Physiological Laboratory, Trinity College, Dublin.

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The formation of urea from ammonium carbonate in aqueous solutions at temperatures below 50° is a reaction of considerable biochemical interest in connection with the obscure mechanism of the formation of urea in the animal body.

Under these conditions urea has been obtained from ammonium carbonate or bicarbonate by a variety of apparently unrelated methods, which may be summarised as follows.

1. Electrolysis, using an alternating current. Drechsel [1880].
2. Electrolysis, using a direct current. Millot [1885]; Fichter, Steiger and Stanisch [1918].
3. Zymolysis, using concentrated preparations of urease. Kay [1923]; Mack and Villars [1923].
4. Incubation with various charcoals. Fichter and Kern [1925].
5. Incubation in a sealed tube for 12 months. Werner [Fearon, 1926].

As a result of the present investigation it has been found possible to detect urea in concentrated solutions of ammonium carbonate after ultra-violet irradiation for periods of 2–10 hours. This is in agreement with the conclusions of Bailey, who detected traces of urea after the exposure of gaseous mixtures of ammonia and carbon dioxide to radium emanation [1925] and to ultra-violet irradiation [1927].

Experimental.

Reagents and apparatus.

1. The ammonium carbonate employed was the purest form supplied by Kahlbaum. It was not analysed for carbamate or bicarbonate.
2. The xanthhydrol was obtained from Poulenc. It was used in 10 % concentration in methyl alcohol.
3. The urease used was the Dunning preparation.
4. The source of irradiation was a Hanovia "Alpine Sun" A.C. mercury vapour lamp. To compensate for any decrease in the efficiency of the lamp the quartz arc tube was replaced by a new one early in the investigation.

Method.

The solutions were irradiated in a flat porcelain dish, 21 cm. in diameter, on account of the observation made by Fichter and Kern [1925] that the prolonged action of warm, concentrated ammoniacal solutions is liable to extract silicic matter from glass-ware, and thus interfere with the specificity of the xanthhydrol test. The distances of the solutions from the lamp were such that the temperature of the liquid did not rise above 45°. In most of the experiments the temperature range was 30°–40°.

After irradiation, the solutions were concentrated to about 5 cc. on a water-bath, and either tested directly by means of urease, or treated with four volumes of glacial acetic acid and filtered free from dust particles. 2 cc. of the 10 % xanthhydrol solution were then added drop by drop and the mixture having been shaken was allowed to stand for 12 hours. Where a precipitate was obtained its identity as dixanthylurea was established by the following tests:

(1) the characteristic sheen of the precipitate, and its microcrystalline nature;

(2) the rosy colour of the warm residue when dried on the filter paper;

(3) the melting-point (260°) as determined in the vapour of diphenyl ether.

Results.

It was found easy to demonstrate the formation of urea in 10 % and 20 % solutions of ammonium carbonate after irradiation of 200 cc. for periods of 2 to 6 hours. The urease test was positive, and the precipitates of dixanthylurea were definite although very small, being of the order of 1–5 mg. per 200 cc.

The control experiments of non-irradiated ammonium carbonate solutions gave no trace of urea after concentration.

250 cc. of a 10 % solution of ammonium carbonate which had been kept in a glass flask exposed to daylight for one year was found to be free from urea.

The effect of various accessory reagents on the reaction was then examined. Glucose, hydroxylamine and formaldehyde were selected as reducing agents; methylene blue, ammonium nitrate and hydrogen peroxide as oxidising agents; copper, iron, and uranium as possible metallic catalysts; methyl orange, malachite green, and eosin as possible photo-catalysts.

The principal results are summarised in Table I.

Of all the accessory reagents examined, malachite green had the most definite effect in increasing the yield of dixanthylurea. During the irradiation the solutions effervesced in a manner not observed with any of the other dye-stuffs examined. Accordingly, these experiments were repeated with controls, and the weight of dixanthylurea determined. After irradiation, the unchanged dye-stuff was removed by acidifying the concentrated solution with acetic acid and extracting with chloroform.

Exps. 3 and 4 are controls, and indicate that the urea did not originate from the action of the ammonium carbonate on the dye-stuff in the absence of the irradiation, or from the irradiation of the dye-stuff in the absence of the ammonium carbonate.

Table I. *Irradiation of ammonium carbonate.*

Time, 2 hours. Temperature, 10°—40°. Distance from lamp, d cm.

Expt.	$(NH_4)_2CO_3$ g.	H_2O cc.	d cm.	Urea	Accessory reagent
1	10	100	25	+	None
2	20	200	15	+	None
3	36	120	15	+ +	None
4	8	100	25	+	Glucose, 2 g.
5	20	100	15	−	$NH_2OH.HCl$, 1 g.
6	40	200	10	−	NH_4NO_3, 1 g.
7	40	200	20	+ +	(Copper stirrer)
8	40	200	10	+	Uranium acetate, 0·2 g.
9	20	100	10	+	Eosin, 0·001 g.
10	40	200	10	+ + +	Malachite green, 0·04 g.
11	40	200	10	+	Methyl orange, 0·03 g.
12	10	100	25	−	Methylene blue, 0·01 g.

Table II. *Photocatalytic action of malachite green.*

Time, 10 hours. Temperature, 10°–40°. Distance from lamp, d cm. Vol. of solution, 200 cc.

Expt.	$(NH_4)_2CO_3$ g.	Dixanthylurea g.	Malachite green g.	d
1	40	0·061	0·02	10
2	40	0·046	0·01	8
3	40	0·000	0·01	infinity
4	0	0·000	0·01	10

Discussion.

In the absence of more adequate data it appears premature to discuss the possible mechanisms concerned in these low temperature syntheses of urea. A general conclusion is that urea is a stable end-product which may be formed in various ways in systems containing ammonia and carbonic acid. Two such ways are (1) alternate reduction and oxidation of carbamic acid, amounting to indirect dehydration; and (2) direct dehydration of carbamic acid. The former process offers an explanation of the production of urea during the electrolysis of solutions of ammonium carbonate, and also, it is believed, is concerned in the photosynthesis of urea.

The latter process, direct dehydration of carbamic acid, is probably responsible for the synthesis of urea by the action of urease, active charcoal, or incubation in a sealed tube.

No attempt was made in the present investigation to determine the range of the ultra-violet spectrum concerned in the synthesis of urea. It was observed, however, that the efficiency of the quartz lamps decreased notably during a lengthy series of experiments, and eventually the photosynthetic properties became negligible[1].

[1] Note added Sept. 18th, 1927. By means of the ultra-violet spectroscope it was found that the effective radiations were between 250 $\mu\mu$ and 200 $\mu\mu$. When a glass screen was interposed, or when the lamp had aged sufficiently, the radiations did not extend into the ultra-violet beyond 250 $\mu\mu$. Under these conditions no urea could be obtained photosynthetically.

SUMMARY.

1. Urea has been produced by the ultra-violet irradiation of solutions of ammonium carbonate at temperatures not exceeding 45°.

2. The yield of urea was increased by the presence of malachite green, but not by that of methylene blue, methyl orange, or eosin.

3. The effective radiations for the synthesis of urea lie between 250 $\mu\mu$ and 200 $\mu\mu$.

REFERENCES.

Bailey (1925). *Proc. Roy. Dub. Soc.* (N.S.), **8**, 165.
—— (1927). Private Communication.
Drechsel (1880). *J. prakt. Chem.* **22**, 476.
Fearon (1926). *Physiol. Rev.* **6**, 399.
Fichter and Kern (1925). *Helv. Chim. Acta*, **8**, 301.
Fichter, Steiger, and Stanisch (1918). *Z. Elektrochem.* **24**, 41.
Kay (1923). *Biochem. J.* **17**, 277.
Mack and Villars (1923). *J. Amer. Chem. Soc.* **45**, 501.
Millot (1885). *Compt. Rend. Acad. Sci.* **101**, 432.

CXLV. THE EFFECT OF CERTAIN SUGAR DERIVATIVES ON INSULINISED MICE.

By ALEXANDER HYND.

From the Physiology Department, University of St Andrews.

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In their researches on the efficiency of various sugars and their derivatives in relieving the symptoms caused by insulin in mice, Herring, Irvine and Macleod [1924] made no use of nitrogenous derivatives. Consequently, early in the spring of 1925, a series of experiments was carried out to test the effect of such compounds, but the results then obtained were not reported owing to their negative character. However, as, recently, other workers have published researches covering the same field, the following results are now communicated.

As a large number of the better known nitrogenous sugar derivatives contain their nitrogen in a group which is foreign and toxic to the animal organism, the compounds investigated were limited to glucose derivatives, in which the nitrogen was present either as an NH_2-group or in the form of urea.

In carrying out the experiments the technique adopted was identical with that described by Herring and co-workers, except that control experiments with normal mice were carried out with each of the compounds tested. These were: (1) glucosimine, (2) glucose ureide, (3) glucosamine hydrochloride, and (4) chitose. A 15–20 % solution of each was employed, 5–10 minims being used for an injection.

(1) d-*Glucosimine.*

The glucosimine used for the first experiments was prepared by Lobry de Bruyn's method [1895], and, as the melting point of the specimen agreed with that quoted (127–128° with decomposition), the material was taken as sufficiently pure for use. Its injection into mice rendered comatose by insulin brought about slight and temporary relief, but this was subsequently shown to be due in all probability to a slight contamination with glucose since a specimen of glucosimine (M.P. 129°), prepared by Ling and Nanji's method [1922] and then recrystallised from methyl alcohol, not only was incapable of producing any relief, but seemed to exert a toxic action.

The specific rotation of the first specimen of glucosimine was found to be + 23·61° ($c = 2{\cdot}287$), that of the purified glucosimine was + 19·87° ($c = 1{\cdot}928$), which agrees with that quoted by Lobry de Bruyn, namely + 19·5°. Consequently, a specimen of glucosimine, although possessing the required melting point, cannot be taken as reliable unless it is known that the $[\alpha]_D$ is less than + 20°.

(2) d-*Glucose ureide.*

The glucose ureide employed was perfectly pure (M.P. 207°, $[\alpha]_D = -23{\cdot}44°$) [Hynd, 1926]. This compound had no effect on either normal or insulinised mice.

(3) d-*Glucosamine hydrochloride.*

No symptoms were detected on injecting the pure salt into normal mice. Four mice suffering from insulin convulsions were in no way benefited by injection of this compound, but recovered slowly after the administration of glucose. Moschini [1924] has recorded similar findings. As this worker found that a previous injection of this compound diminished and delayed the effect of insulin on rabbits, he concluded that glucosamine is transformed slowly into glucose *in vivo*. This appears to be opposed to the generally accepted view as to the behaviour of glucosamine in the animal organism, a summary of which is given by Levene [1925]. An alternative explanation is that glucosamine hydrochloride, just like ammonium chloride, would tend to produce an acidosis, which would render the rabbit somewhat more resistant to the action of insulin [Abderhalden and Wertheimer, 1924; Hetényi, 1926].

(4) *Chitose.*

Chitose syrup was tested on one normal and on six insulinised mice. It was quite inactive.

Discussion.

Despite the negative character of the above results, they are not without interest when the close relationship of all four compounds to glucose is considered. In both glucosimine and glucosamine, the glucose molecule is altered only by the substitution of an NH_2-group for an OH-group. Yet this is sufficient to render the molecule inactive.

As all the evidence points to the reducing group being essential, its substitution in the case of glucose ureide and glucosimine made it unlikely that these derivatives would have any efficiency. However, these two compounds differ from those hitherto examined with the reducing group substituted, in that they are relatively unstable in alkaline solutions, and might, therefore, have been expected to produce a positive result under the conditions obtaining in the animal body.

The third nitrogenous compound employed, namely glucosamine, conforms to the general formula arrived at by Irvine for a compound which should alleviate the symptoms produced by insulin, as the only alteration is at the second carbon atom. The groups at this position have so far been regarded as immaterial, but it is evident that while it is permissible to have inversion of the OH-group at the second carbon atom, this OH-group cannot be substituted without destroying the efficiency of the compound.

The fourth compound examined, namely chitose, is nitrogen-free, but again closely related as it is obtained by the deamination of glucosamine,

and may be regarded as 2.5-anhydro-glucose (or -mannose). Chitose is thus the third example of an anhydro-sugar which has been tested as to its efficiency for relieving insulin symptoms. Winter [1926] carried out experiments with α-glucosan, while the action of β-glucosan was investigated at an earlier date by Herring, Irvine and Macleod [1924]. Negative results were obtained in both these cases, presumably because the reducing group was absent. The inactivity of chitose cannot be explained in this way, as the reducing group is free. Further, as the correct configuration is preserved, the lack of efficiency must be referred to the ring structure which is present in the compound.

It is clear that glucose is by far the most efficient sugar for counteracting the action of an excessive amount of insulin, and any alteration of its molecule, except changes at the second carbon atom to give either mannose or glucal, which is converted readily into mannose [Winter, 1926], lowers the efficiency. Though changes at the second carbon atom are involved in glucosamine and chitose, the non-activity of these two compounds is not surprising. Accordingly it may be concluded that the introduction of nitrogen (*e.g.* glucosimine, glucosamine and glucose ureide) or of a second ring structure (*e.g.* chitose, α-glucosan and β-glucosan) into the glucose molecule completely destroys the power of relieving insulin hypoglycæmia.

REFERENCES.

Abderhalden and Wertheimer (1924). *Pflüger's Arch.* **205**, 559.
Herring, Irvine and Macleod (1924). *Biochem. J.* **18**, 1023.
Heténi (1926). *Klin. Woch.* **5**, 800.
Hynd (1926). *Biochem. J.* **20**, 205.
Levene (1925). Hexosamines and Mucoproteins, Chap. IX (Longmans, London).
Ling and Nanji (1922). *J. Chem. Soc.* **121**, 1685.
Lobry de Bruyn (1895). *Rec. Trav. Chim.* **14**, 98.
Moschini (1924). *Arch. ital. Biol.* **74**, 117 from *Chem. Zentr.* 1926, **1**, 2113.
Winter (1926). *Biochem. J.* **20**, 668.

CXLVI. THE ANTAGONISM OF GLUCOSONE AND CYANIDES *IN VIVO*.

By ALEXANDER HYND.

From the Physiology Department, University of St Andrews.

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Recently many references have appeared in the literature to the antagonistic action towards cyanides which results from the previous administration of glucose either alone, or in conjunction with insulin. As, in the course of experiments on the physiological action of glucosone [Hynd, 1927], it had been noticed that an antagonism also appeared to exist between glucosone and potassium cyanide, the question was investigated more fully in the hope of correlating the results referred to above.

Owing to the small amount of glucosone available, the majority of the experiments involving the use of this compound were carried out on mice, but a certain number were performed on young albino rats and also on rabbits. In the experiments described 67 mice, 14 rats and 14 rabbits have been employed.

I. The subcutaneous injection of potassium cyanide into mice.

In the first experiments potassium cyanide was administered subcutaneously. The mice used were found to be much more resistant to this poison than was expected, and consequently a number of preliminary trials had to be made to determine the necessary dosage for the object in view. From these it appeared that 4 minims of a 0·5 % solution of potassium cyanide was a suitable dose for a mouse weighing 20 g. Such a dose of cyanide rarely renders a mouse moribund, but nevertheless it produces, in a short time, very definite symptoms, usually the first to be noted being a slight retraction of the head and "biting at the air." Within five minutes after the injection leg-weakness is noticeable, and this soon becomes more marked so that in the course of another five minutes the animal is unable to walk—any attempt to do so being followed by severe tremors. Handling of the mouse at this stage also induces tremors, after which the animal sometimes rolls over and lies on its back. Return to the prone position may not occur for some time, but it usually takes place suddenly, and is sometimes accompanied by a convulsion. Thereafter recovery takes place slowly and the mouse appears fairly normal in about two hours after the injection.

Three mice of approximately equal weight were selected for each experiment—the first being injected with cyanide, the second with a suitable amount of glucosone, and the third receiving injections of the same amounts of both cyanide and glucosone.

An 11·35 % solution of glucosone was used and 0·3 of a minim per g. body-weight was the amount injected, as such a dose was known to be non-lethal but capable of producing severe symptoms. This always was realised in the animals receiving glucosone alone—marked sprawling of the legs being evident in 5 to 10 minutes after injection and more severe symptoms in the course of 15 to 20 minutes.

Animals receiving injections of both glucosone and cyanide were from 20 to 30 minutes before exhibiting any symptoms. Usually a slight glucosone effect, such as sprawling of the legs, was observed 20 minutes after the injection instead of in 5 minutes in the control animals, and no cyanide effect was detected till 30 minutes had elapsed. The symptoms displayed were of a mild character and of a mixed type, at one moment suggesting the cyanide, at another the glucosone action, but return to the normal required a somewhat longer period than was the case with a mouse receiving potassium cyanide alone.

This experiment has been repeated on three separate sets of animals with uniform results.

II. The subcutaneous injection of acetonitrile.

A series of experiments was then carried out in which the alkali cyanide was replaced by acetonitrile. As the result of preliminary experiments it was found that a dose of 0·2 minim of a 5 % solution of acetonitrile per g. of body-weight produced in a mouse a fairly characteristic train of symptoms. These included tremors, lowering of body temperature, sprawling limbs and convulsions.

As before, three mice of approximately the same weight were used in each experiment—the first being injected with acetonitrile, the second with acetonitrile and glucose, and the third with acetonitrile and glucosone. All the animals used in this set of experiments exhibited in about 20 minutes marked sprawling of both fore and hind limbs, while at a later stage those not receiving glucosone, as a rule, rolled over on their backs and lay clasping the fore limbs together. The "glucosoned" mice showed no sign of tremors, which was a striking feature in the other animals. Moreover, though on some occasions, half an hour after being injected, the mice which had received glucosone appeared most affected, after 3 to 4 hours they were quite normal, whereas the others still exhibited effects of the nitrile (including diarrhoea) and, in some cases, the mice injected with acetonitrile alone were found dead the following morning.

III. The effect of an atmosphere containing hydrogen cyanide.

The effect of the inhalation of hydrogen cyanide was then studied on mice. For this purpose a bell-jar of 7 l. capacity, closed with a ground glass plate and fitted with a hydrogen cyanide generator, was used—the latter being so arranged that the hydrocyanic acid was liberated only after the experimental animals had been introduced into the bell-jar. By trial it was found that the gas evolved from the interaction of 3 cc. of 4 % potassium cyanide and 2 cc. of 40 % sulphuric acid, furnished a suitable concentration of hydrogen cyanide. Although the exact concentration of the hydrogen cyanide was unknown, this was immaterial, as three animals were used in each experiment—the first injected with a non-lethal dose of glucosone, the second with an equivalent amount of glucose, and the third not injected. Animals of equal weight were chosen as far as possible, and where this could not be managed, the lightest received glucosone and the heaviest was used as the normal animal. Eight experiments of this type have been carried out and invariably it has been found that glucose affords no protection against hydrogen cyanide. On the other hand, a subcutaneous injection of glucosone enables a mouse to resist the toxic action of hydrogen cyanide. These facts are clearly demonstrated in the following summary of a more or less typical experiment.

Effect of an atmosphere containing hydrogen cyanide.

Time a.m.	Black mouse 20·0 g.	White mouse 19·4 g.	Grey and white mouse 19·6 g.
10.9	No injection	3 minims 18·13 % glucosone	3 minims 20 % glucose
10.15	HCN atmosphere	HCN atmosphere	HCN atmosphere
10.16	Running about sniffing	Sitting with head down	Appears normal
10.19	Respiration very rapid: beginning of cyanide effect	Slight sprawling of legs: due to glucosone	Appears drowsy
10.24	Leaping about	Slight sprawling of legs: due to glucosone	Walking about
10.28	Sitting gasping: still able to cling	Still sprawling but can walk about when disturbed	Beginning to show effect of cyanide
10.30	Lying on side: gasping	Slightly improved but eyes protruding	Violent convulsion: then rolls over on to its side
10.32	Symptoms very severe: slight convulsion	Slightly improved but eyes protruding	Respirations very slow
10.34	Rolls over: several severe convulsions	Slightly improved but eyes protruding	Eyes protruding
10.52	Lying moribund	Beginning to show slight cyanide effect	Dead
	Removed to ordinary air.	*Removed to ordinary air.*	
10.56	Severe convulsions	Sitting in normal position but unwilling to move	
11.0	Slightly improved	Definitely improved	
11.5	Still blind: severe tremors when handled	No tremors, but signs of diarrhoea	
11.10	Several convulsions	Fairly normal	
11.30	Improving generally: eyes normal	Fairly normal	
p.m. 2.0	Practically normal	Drowsy: fur ruffled	

Certain of the experiments carried out have been even more striking than the above, as, for example, that in which a normal mouse of 26 g. weight was rendered moribund in 10 minutes by the hydrogen cyanide vapour, while a 20 g. mouse, which had been injected with glucosone, showed only slight symptoms after 2½ hours' inhalation, at which time the normal mouse died.

Very similar results have been obtained in three experiments with young albino rats of an average weight of 35 g. and from the same litter, and also in one experiment when the average weight was 75 g. All the rats used in these experiments survived, but the striking feature was the rapidity with which those injected with glucosone became normal on removal from the atmosphere of hydrogen cyanide. The recoveries of both the "normal" rat and that injected with glucose were in comparison slow, and moreover were accompanied by a number of severe convulsions.

A number of experiments were also carried out to determine whether insulin with or without glucose furnished any protection for mice exposed to the vapour of hydrogen cyanide. Doses of insulin varying from 0·8 to 1·6 units per 100 g. body-weight were employed, but no definite protective action could be demonstrated. In fact all the insulinised mice, which were exposed to gaseous hydrocyanic acid, died, while normal mice, though displaying severe cyanide symptoms as the result of the same exposure, recovered on removal to ordinary air. In the majority of the experiments it appeared that exposure to the atmosphere of hydrogen cyanide hastened the development of the insulin symptoms, but this point could not be established by control experiments.

IV. Experiments involving the intravenous injection of potassium cyanide into rabbits.

(i) *The antagonistic action of insulin plus glucose.*

(*a*) A rabbit of 2·7 kg. was injected intravenously with 30 units of insulin and 4 cc. of a 40 % solution of glucose. Eight minutes later, 5 minims of a 4 % solution of potassium cyanide were injected into the same ear vein. Immediately the animal showed difficulty in breathing and, as soon as released, it rolled over on its side. The head was retracted and respirations were very slow, but in 2 to 3 minutes they improved slightly. This was followed a minute later by a severe convulsion, and two minutes afterwards the animal was able to sit up, although the hind limbs were still extended. Ten minutes later the rabbit looked quite normal and no further symptoms were noticed during the course of the day.

Two days later, using the same rabbit, the experiment was repeated except that no insulin was administered. The animal behaved exactly as on the previous occasion—severe symptoms immediately after the cyanide injection and return to the normal in about 10 minutes.

(*b*) The above experiment was repeated with a larger dose of cyanide (8 minims of 4 % KCN) using two rabbits weighing 2720 g. and 2940 g.

respectively—the former, a male, receiving 30 units of insulin and the latter, a female, no insulin. They behaved in exactly the same manner, except that after returning apparently to the normal condition the insulinised male rabbit showed slight insulin symptoms but recovered without any treatment.

(*c*) Having found that the intravenous injection of 5 minims 10 % potassium cyanide into a rabbit (♂) weighing 3 kg. caused death almost instantaneously, another rabbit (♀) of the same weight was injected intravenously with 4 cc. of 40 % glucose and 30 units of insulin, and five minutes later 5 minims 10 % potassium cyanide were introduced into the marginal vein of the other ear. Severe symptoms ensued immediately, but 20 minutes later the animal was fairly normal, its behaviour having been very similar to that of the first rabbit described above in experiment (*a*).

Five days later this experiment was repeated on the same animal with identical results. After another period of five days the same rabbit was used to study the effect of the same amount of cyanide after glucose but without insulin. Complete though somewhat slow recovery took place, the rabbit being able to sit up in the normal position 45 minutes after the injection. About a fortnight later, on injecting the same amount of cyanide alone, this rabbit collapsed at once and died in one and a half minutes.

(ii) *The antagonistic action of glucosone.*

(*a*) A young rabbit weighing 900 g. was injected intravenously with 1·5 cc. of 18·13 % glucosone[1], and then immediately afterwards with 3 minims of 5 % potassium cyanide. Severe struggling took place for about half a minute, thereafter the animal lay on its side quite collapsed. After one minute the corneal reflex returned. No twitching or tremors were noticed, but the rabbit lay quite limp for about ten minutes, at which time it was able to hold up its head, although the limbs remained limp till about five minutes later. Twenty minutes after receiving the injection, the animal appeared drowsy but otherwise normal.

A week later the effect of the same dose of cyanide was studied on the same rabbit. As before severe struggling took place, followed by collapse, the animal lying on its side helpless, with head retracted and eyes fixed. Respirations, which at first were slow, gradually improved in the course of 2 to 3 minutes, during which time a number of inspiratory cries were uttered. Five minutes elapsed before the reflexes returned: in ten minutes, after marked twitching especially of the fore limbs, an attempt was made to sit up, but after doing so the rabbit gradually fell over again on its side. Two minutes later, however, it succeeded in resuming the normal position but was not quite recovered in half an hour, being disinclined to move, sitting with ruffled fur and having a general scared appearance.

[1] So far the physiological action of glucosone has been reported only on mice [Hynd, 1927]. Its action on other species has been investigated and an account of this is at present in preparation.

(*b*) Two young rabbits of the same litter and of approximately the same weight were used for the next experiment. One (530 g.), on injection with 1·6 minims of 5 % sodium cyanide, displayed the usual symptoms and died in 1½ minutes. The other (550 g.) was given intravenously 1 cc. of 18·13 % glucosone and five minutes later 1·6 minims of 5 % sodium cyanide. Less severe struggling ensued and the animal died within a minute of receiving the cyanide.

(*c*) In the following experiment the dosage of cyanide was reduced by half, and two rabbits of the same weight (620 g.) and from the same litter were employed.

20 minims tap-water mixed with 1·7 minims 2·5 % sodium cyanide were injected into the left ear vein of one animal. After half a minute's violent struggling the rabbit lay collapsed on its side. Improvement in respiration was noted in three minutes, but reflexes did not return for 11 minutes. Efforts to hold the head erect were not made until 18 minutes had elapsed, and at this time both fore and hind limbs were still sprawling. Return to a fairly normal condition required about 50 minutes.

20 minims 18·13 % glucosone mixed with 1·7 minims 2·5 % sodium cyanide were similarly injected into the other rabbit. Struggling was not nearly so marked and the animal never collapsed. Reflexes were never absent, the head was kept always erect, but the limbs were extended for a few minutes. Three minutes after the injection the rabbit seemed fairly normal but looked frightened.

Two days later the treatment of the animals was reversed. On giving the same dose of glucosone and cyanide to the first rabbit slight struggling occurred during the injection but otherwise no effect was noticed. The second rabbit, on the other hand, struggled so violently during the injection of cyanide alone that only half the amount was introduced and the animal died within a minute.

Three days later, similar behaviour occurred with the surviving rabbit when it was injected with 1·7 minims of 2·5 % sodium cyanide previously diluted with 18 minims of tap-water. After very violent struggling during the injection, the animal collapsed and died almost instantaneously.

Discussion.

The view expressed by Horvath [1926] that the therapeutic value of glucose consists in part in an antitoxic rôle toward the CN-group, is not borne out by the results of the experiments now described. In no case has there been observed any definite protective action of glucose against alkali cyanide, gaseous hydrocyanic acid, or acetonitrile. This is also contrary to the findings of Violle [1926], who has stated that, while a concentration of 2 g. hydrogen cyanide per cubic metre of air will kill a rabbit in a few minutes, animals previously fed or injected with glucose can breathe this toxic atmosphere for more than an hour without ill effects. As the concentration of hydrogen

cyanide used in his experiments was of the same order as that employed by the present author, there is the possibility that the divergence in the results might be explained by the fact that the experiments were conducted on a different species of animal, for, while Violle used rabbits, my comparable experiments were confined to mice and rats. This explanation, however, seems unlikely as already Voegtlin, Johnson and Dyer [1926] and also Heymans and Soenen [1927], who used rabbits and guinea-pigs, have described experiments, the results of which point to glucose being incapable of counteracting the toxic effects of cyanogen compounds.

On the other hand, the results of the present investigation point to the fact that glucosone and potassium cyanide are antagonistic in their actions in the animal organism, for the symptoms accruing from either compound are delayed in their development and diminished in their intensity when simultaneous injections of these substances are given subcutaneously.

Evidence that glucosone counteracts the CN-group is also furnished by the experiments with acetonitrile. These were performed in the hope that this somewhat less toxic compound would yield even more definite results than those obtained with the alkali cyanide. This was not realised, however, and, in fact, the results were somewhat less definite.

Although controls were always carried out against experiments involving the subcutaneous injection of cyanides, it was difficult, especially when dealing with small animals such as mice, to be certain that the proper amount was injected, and even though this operation had been accomplished successfully, there was no guarantee that the rate of absorption would be comparable in the experimental and the control animal. However, any objection on this account is negatived owing to the number of experiments carried out and the uniformity of the results obtained.

Further evidence of a convincing character, as to the antagonistic action of glucosone and cyanide, is furnished by the results of the experiments in which the experimental animals were exposed to an atmosphere containing hydrogen cyanide. Though very definite results were obtained in these inhalation experiments with both mice and rats, the dose of glucosone required to afford protection is a fairly large one, being only slightly less than what would be a lethal dose under normal atmospheric conditions. This may explain why insulin with or without glucose, or even glucose itself, has been found to be useless for small animals, as sufficient glucosone would not be produced quickly enough to counteract the large amount of cyanide. The author's suggestion [Hynd, 1927] that insulin converts inactive glucose into utilisable glucosone, may provide the key for the explanation of the results reported by Szolnoki [1926] and by Rosenberg [1926] regarding the antidotal action of insulin in hydrocyanic acid poisoning.

Reference to the experimental part dealing with the intravenous injection of potassium cyanide into rabbits will show that attempts to repeat the experiments described by Rosenberg [1926] were not altogether successful, as in the

third experiment the rabbit recovered from the cyanide injection when protected by glucose without insulin. The symptoms which ensue after an intravenous injection into a rabbit of an amount of cyanide approaching the lethal dose are so severe that recovery of the animal appears to be always rather problematic, no matter what substance has been administered for its protection. So large a number of factors are involved, as for example, the rate at which the cyanide is injected and its subsequent distribution, that one must hesitate in drawing too definite conclusions.

Nevertheless, taking the results of the present experiments as a whole, it seems established that glucosone exerts an antagonistic action towards the CN-group. In this respect, however, glucosone is not so efficient as, for example, cystine, with which it was compared and which, as Voegtlin and his co-workers have shown, is able to protect rats against lethal doses of cyanide. Consequently, glucosone in no sense can be recommended as an antidote in hydrocyanic acid poisoning, and evidently the use of glucose or of glucose plus insulin would be of even less avail for such a purpose.

It is now generally accepted that the normal mechanism of detoxicating cyanides in the animal body involves interaction with sulphur compounds. Although this may be true under normal conditions, it seems possible that, when excessive amounts of cyanides have to be dealt with, the supply of such thio-compounds may be inadequate and carbohydrate may then be called upon, just as glycuronates appear in the urine when there is produced an excess of compounds which normally are eliminated as ethereal sulphates. As in glucosone, an oxidation product of glucose, the body would have at its command an efficient detoxicating agent, it seems possible, in view of the results already obtained [Hynd, 1927], that glucosone is a compound of real physiological importance.

SUMMARY.

1. In the case of mice and rats, a subcutaneous injection of glucose, or of glucose plus insulin, affords no protection against the toxic action following (*a*) the subsequent subcutaneous injection of either alkali or methyl cyanides, or (*b*) the inhalation of gaseous hydrogen cyanide.

2. Under the same experimental conditions, glucosone exerts a definite antagonistic action towards cyanides, but is less efficient than cystine.

3. The previous, or simultaneous, intravenous administration of glucosone, or of glucose plus insulin, appears to be protective against the intravenous injection of alkali cyanide into rabbits, but the results obtained are somewhat variable.

REFERENCES.

Heymans and Soenen (1927). *Compt. Rend. Soc. Biol.* **96**, 202.
Horvath (1926). *Japan Med. World*, **6**, 133.
Hynd (1927). *Proc. Roy. Soc. Lond.* B, **101**, 244.
Rosenberg (1926). *Med. Klin. Berlin*, **22**, 1650.
Szolnoki (1926). *Deutsch. med. Woch.* **52**, 1427.
Violle (1926). *Bull. Acad. Med.* **95**, 664.
Voegtlin, Johnson and Dyer (1926). *J. Pharm. Exp. Ther.* **27**, 467.

CXLVII. THE SYNTHESIS OF ANTINEURITIC VITAMIN BY YEAST.

By GEOFFREY LEWIS PESKETT.

From the Rockefeller Department of Biochemistry, Oxford.

(*Received August 19th, 1927.*)

Recently Hawking [1927] has shown that yeast can synthesise the factor which cures the symptoms of head retraction in pigeons fed upon polished rice. These experiments were not quite conclusive on two accounts, viz.: (1) the amount of "bios extract" used in growing the yeast contained so much of the antineuritic factor that the amount of the latter which was synthesised appeared to be small; and (2) there was some contamination of the yeast by bacteria. The following experiment was performed under conditions which were more satisfactory in these respects.

Experimental.

The cane sugar, salts and "bios extract" were precisely the same as Hawking employed, and the experiment may be regarded as a continuation of his work. A basal medium was used, of which the composition was:

KH_2PO_4	5·0 g.	$CaCl_2$	0·25 g.
NH_4Cl	2·5 g.	Cane sugar	50·0 g.
$MgSO_4$	0·35 g.	Glass-distilled water to	1000 cc.

The yeast was the same strain of *Saccharomyces cerevisiae*, and the stock cultures were grown at 25° in 5 cc. portions of basal medium containing 0·05 cc. of "bios extract." A small loopful of stock culture (21 days old) was transferred to a fresh tube of this medium and grown at 25° for one day. The supernatant fluid was pipetted off and replaced by 5 cc. of basal medium, the resulting suspension being used for inoculation of the flask in the following experiment.

Experiment. 0·5 cc. of "bios extract" was added to 1500 cc. of basal medium in a 2-litre flask, the neck of which had been drawn out to a diameter of 15 mm. in order to minimise the risks of accidental infection. After sterilisation (by steam-heat on three successive days) the flask was inoculated w th approximately 3000 yeast cells and maintained at 25° for 10 days with occasional shaking. Samples were then removed to agar slopes, and the culture was at the same time examined in wet and dry films. The latter showed that there was no bacterial contamination, and the growth on the agar slopes consisted in all cases of pure yeast. The greater part of the fluid was syphoned

off, and the remainder separated on the centrifuge. The resulting yeast weighed 5·7 g. (wet). This yeast was immediately extracted by boiling for one minute with 7 cc. of 1 % acetic acid, and the extract separated from the cell-residue by centrifuging. The fluid was decanted, made up to a volume of 10 cc. with water, and divided into two equal parts.

Two pigeons were used, each receiving 5 cc. of the extract. In both the symptoms of head retraction disappeared, to reappear in one case after 2·75, and the other after 3·25 days. The yeast therefore contained at least 6 day doses.

Hawking's experiments showed that the "bios extract" did not contain more than 0·7 day doses in 0·5 cc. The cells used for inoculation represented a thousandth part of the suspension from which they were obtained, and therefore did not contain more than 0·00007 day doses. Thus, before the development of the yeast, the flask did not contain more than 0·70007 day doses. Of the 6 day doses found in the yeast, 5·3 day doses were presumably synthesised.

SUMMARY.

An experiment is recorded in which 5·7 g. of yeast were produced in a medium which did not contain more than 0·7 day doses of antineuritic vitamin. Bacterial contamination was avoided, and the yeast contained at least 6 day doses. Considerable synthesis can therefore occur.

I wish to thank Prof. Peters for his interest and advice, and for his kindness in testing the extract on the pigeons.

REFERENCE.

Hawking (1927). *Biochem. J.* **21**, 728.

CXLVIII. KIDNEY PHOSPHATASE.
II. THE ENZYME IN DISEASE.

By REGINALD THOMAS BRAIN
and HERBERT DAVENPORT KAY (*Beit Memorial Research Fellow*).
From the Medical Unit, The London Hospital, E. 1.

(*Received August 24th, 1927.*)

In an investigation recently described by one of us in this *Journal* [Kay, 1926] data were quoted which strongly suggested a correlation between the phosphatase content of the kidney and the functional activity of this organ.

Further support for this suggestion has now been obtained from a study of the phosphatase content of (*a*) human kidneys, obtained *post mortem* from individuals dying from various causes, including kidney disease, (*b*) kidneys of rabbits with acute experimental nephritis.

Method.

The method used for determining the phosphatase content of a given kidney is that described on pp. 795 and 797 of the communication just mentioned. The activity of the phosphatase of rabbit's, rat's and pig's kidney has been previously shown to have a fairly broad optimum between p_H 8·8 and 9·2. This is true also for human kidney phosphatase. The optimum reaction becomes somewhat less alkaline as the period over which the activity of the enzyme is determined increases. In the great majority of the determinations which are given in the present paper, the activity of each kidney extract has been ascertained at a series of p_H values from 8·25 to 10·0. For two hours' activity the optimum is invariably between p_H 8·8 and 9·2. The unit of kidney phosphatase we define as the amount of enzyme which, at 38°, and in Sørensen's glycine-NaOH buffer solution at p_H 8·9, is required to liberate 1 mg. phosphorus as inorganic phosphate from excess of sodium β-glycerophosphate solution in 2 hours. The phosphatase activity of a tissue we express in units per g. (wet weight). Provided care is exercised in the sampling of the tissue (which should be obtained with as little delay as possible *post mortem*), analyses by different observers on the same kidney give reasonably concordant results (Table I).

Table I. *Kidney phosphatase in units per g. tissue (wet weight).*

	Observer			
No. of expt.	R. T. B.	H. D. K.	R. H. R.	Remarks
1	2·6	2·7	—	Normal
31	0·5	0·4	—	Nephritic
A 241	—	4·7	4·9	Normal
A 192	2·6	2·8	—	Cardiac failure "back pressure" kidney

If a portion of a rabbit's kidney is kept for 24 hours at room temperature before making the determination, there is usually a slight fall (about 10 %)

in the phosphatase activity. The human kidneys used in this series were obtained at the routine *post mortem* examination, usually within 24 hours of death, during which time the cadavers were kept in a cold chamber. With human kidney a further period of 24 hours in the cold box in addition to the usual time between death and *post mortem* examination has no appreciable effect on phosphatase activity. It is therefore unlikely that variations in the phosphatase content of the kidneys, due to *post mortem* changes, exceed 10 % in any of our determinations.

A. Human Kidneys.

Results of determinations carried out on portions of kidney obtained *post mortem* from cases of disease and of accidental death are shown in Tables II, III, IV, and V.

Table II. *Cases without definite clinical or* post mortem *evidence of renal disease or abnormality other than slight parenchymatous degeneration.*

No. of expt.	Age of individual	Cause of death	Report on kidneys *p. m.*	Phosphatase units per g.
A 67	6½ months foetus	Foetus quite healthy. Premature delivery	Normal	1·0
A 80	2 full term foetuses	Delivery	,,	1·8 2·4
A 180	5 hours	—	,,	0·8
17	2 days	Haemorrhage	Anaemia*	1·9
1	6 years	Fractured skull	Normal	2·7
A 241	6 ,,	Injuries	,,	4·7
5	10 ,,	Abscess of brain	,,	4·6
6	Adult	Heart disease	,,	5·4
10	52 years	Injuries	One kidney lacerated, other normal (latter used)	6·2
9	60 ,,	Cerebral tumour	Parenchy. degeneration	3·3
A 11	10 ,,	Injuries	To naked eye, normal	5·8
24	3 months	Diarrhoea and vomiting	Parenchy. degeneration	5·8
25	18 years	Injuries	Anaemia*	6·2
32	35 ,,	Haemorrhage from gastric ulcer	,, *	5·1
A 202	46 ,,	Cerebral tumour	Normal	4·8

* Due to pre-mortal haemorrhage.

Table III. *Cases in which death was due to nephritis.*

No. of expt.	Age of individual	Cause of death	Report on kidneys	Phosphatase activity
2	50	Uraemia	Slightly narrowed cortex	1·6
3	18	,,	Considerable reduction of patternless oedematous cortex	0·4
8	23	,,	Considerable reduction in cortex, pattern moderately clear	0·75
14	48	,,	Broad cortex with indistinct pattern	1·1
23	18	,,	Great reduction in completely patternless cortex	0·7
27	53	..	Great dilatation of pelvis with thinning of renal substance	0·85
29	20	,,	Marked narrowing of cortex. Amyloid infiltration	0·5
31	53	,,	Considerable reduction in width of cortex and greatly blurred pattern	0·45
A 257	46	,,	Typical red granular kidney. Moderate reduction in cortex. Granular distorted pattern	1·0

Table IV. *Cases in which death was due to causes other than nephritis, but in which renal abnormalities were found on* post mortem *examination.*

No. of expt.	Age of individual	Cause of death	Report on kidneys	Phosphatase activity
4	75	Broncho-pneumonia	Atrophied kidneys with a few cysts in cortex	2·2
21	53	Cerebral tumour	Bilateral cystic kidney with cysts throughout cortex and medulla	1·0
7	25	Heart failure	A few cysts and small areas of infarction in cortex beneath the capsule	2·9
30	63	Strangulated hernia. General peritonitis	Slight sub-capsular fibrosis and a few cysts	1·4

Table V. *Cases in which a severe infection was present before death.*

No. of expt.	Age of individual	Description of case	Report on kidneys	Phosphatase activity
11	43	Post-operative broncho-pneumonia	Severe parenchy. degeneration and oedema	4·6
13	50	Pericarditis. Heart failure	Congestion	4·7
15	22	Broncho-pneumonia	Parenchy. degeneration and oedema	2·8
19	14	Lobar pneumonia	Severe parenchy. degeneration	5·7
16	51	Pernicious anaemia. Tuberculosis	Anaemia, parenchy. degeneration and miliary tubercles	3·4
22	41	Broncho-pneumonia. Peritonitis	Anaemia, parenchy. degeneration	2·8
12	46	Hydatid disease of the liver. General peritonitis	Fatty or lipoid degeneration	2·25

Table VI. *Average values.*

Description of cases	Ages	No. of cases	Average value for kidney phosphatase
Normal	2 days and under	5	1·6
Normal, or no evidence of renal disease	3 mths. to 60 yrs.	11	4·8
Chronic nephritics	18 yrs. to 53 yrs	9	0·8
Not nephritic but other renal abnormality	25 yrs. to 75 yrs.	4	1·9
Severe infective conditions	14 yrs. to 51 yrs.	7	3·7

Table VI summarises the first series of findings:

(*a*) Foetal and very young kidney tissue is considerably less active than older tissue.

(*b*) In patients with chronic nephritis dying from uraemia, the phosphatase content of the kidney is markedly diminished, being reduced, on the average, to about one-sixth of the normal adult value.

(*c*) In cases in which death was not due to nephritis, but in which degenerative conditions were found in the kidneys *post mortem*, the phosphatase content of the kidney is also much reduced.

(*d*) The kidneys from cases in which a severe infective process was present before death, show some diminution in phosphatase content which is, however, much less marked than in (*b*) or (*c*) above.

B. KIDNEYS OF RABBITS WITH ACUTE EXPERIMENTAL NEPHRITIS.

Acute experimental nephritis was induced in rabbits by subcutaneous or intravenous injection of lethal doses (from 1·3 to 4 mg. U per kg.) of uranyl nitrate [cf. MacNider, 1920]. Death usually ensued from 4 to 7 days after the injection, and the kidneys were examined as soon as possible *post mortem*. Together with cardiac dilatation, there was invariably severe generalised oedema with large exudates of clear fluid into the peritoneal and pleural cavities. The kidneys were swollen and oedematous, but there was little if any change in the relative breadth of the cortex to that of the medulla.

In addition to the phosphatase content, the ratio dry weight to wet weight was determined in a number of cases, in order that the numerical correction of the phosphatase content for the "dilution" of the kidney tissue with oedema fluid might be ascertained.

The results are shown in Table VII.

Table VII.

Kidneys of control rabbits			Kidneys of animals with experimental nephritis				
No. of expt.	% dry wt.	Phosphatase activity	No. of expt.		% dry wt.	Phosphatase activity	Summary of histological report on kidneys*
219 (1 animal)	—	8·1	219 (2 animals)	A	—	2·9 L.	In all cases a severe degeneration of the tubules, and to a less extent of the glomeruli was found. In 219 B glomerular damage was relatively trivial. The order of severity of damage in these four animals based on histological examination was as follows: (1) 221 A (R) (2) 221 B (R) (3) 219 A (R) (4) 219 B (R)
					—	3·1 R.	
				B	—	3·3 L.	
					—	3·7 R.	
221 (2 animals)	23·9	10·9 L.	221 (2 animals)	A	17·5	2·4 R.	
	22·9	12·5 R.			17·6	2·3 L.	
	24·0	12·6 L.		B	16·1	2·5 R.	
					15·6	2·2 L.	
228 (1 animal)	23·8	10·7 L.	228 (1 animal)		15·7	2·8 L.	Severe degeneration and necrosis of the tubules
	22·4	— R.			16·0	3·3 R.	
238 (1 animal)	—	12·6	238 (1 animal)		—	3·4 L.	Not examined
					—	3·4 R.	

L. = left kidney; R. = right kidney.

* We are much indebted to Dr D. S. Russell for her kindness in making a histological examination of these kidneys.

From Tables VI and VII it will be seen that severe kidney damage, whether brought about by a protracted process of disease or induced rapidly by a toxic agent, is associated with a very large fall in the phosphatase content of the tissue. The observed diminution far outweighs any decrease which the "dilution" of the tissue by oedema fluid might bring about. Thus taking Exp. 221, Table VII, the average dry weight of the normal kidney was 23·6 % of the wet weight, giving an average phosphatase content of 50·5 units per g. dry weight. For the nephritic kidneys, with an average dry weight of 16·7 % of the wet weight, the phosphatase content per g. dry weight is only 23·5 units.

From Table VII it will also be seen that the order of increasing severity of damage, as determined histologically by a second observer, agrees with the order of decreasing phosphatase content per g. of tissue.

In the previous communication, already cited, we have shown that in the developing animal the phosphatase content of kidney tissue increases with increasing functional activity of this organ. Also, the renal cortex, which contains the essential secretory elements of the kidney, was shown to contain more phosphatase than the medulla. In the present paper, a diminished phosphatase content of the tissue is shown to be associated with diminishing functional capacity resultant from disease. These results suggest that the enzyme phosphatase plays an intimate part in renal activity, either cellular or secretory, and that its presence in amounts greater than a certain minimum is essential for the proper functioning of the organ.

It is admitted that the evidence is circumstantial and indirect, and that we may be dealing here merely with an associated failure of actually independent variables. Thus (*a*) in chronic nephritis the observed diminution in phosphatase activity may be due to the progressive decrease in the number of secretory units and their replacement by scar tissue, leading to a relative and absolute diminution in the amount of the more active cortical tissue; (*b*) in acute experimental nephritis, the poisoning and subsequent destruction of the glomerular and tubule cells would naturally result in the loss of many activities associated with the healthy cells (*e.g.* oxygen uptake, lipase and ereptase activity, glycolytic power), without there being of necessity any direct connection between the activities concerned—in this case between phosphatase activity and secretory ability. Nevertheless, the considerable circumstantial evidence in favour of a more direct relationship between the last two activities is at least not weakened by the results just described.

SUMMARY.

Both in chronic nephritis in man and in acute experimental nephritis in rabbits, the phosphatase activity of the renal tissue is markedly reduced.

The work has been carried out with the aid of a grant from the Research Fund of the London Hospital Medical College.

REFERENCES.

Kay (1926). *Biochem. J.* **20**, 791.
MacNider (1920). *Arch. Int. Med.* **26**, 1.

CXLIX. IDENTITY OF URINARY ALBUMIN.

By LESLIE FRANK HEWITT.

From the Hale and Dunn Clinical Laboratories, London Hospital, E. 1.

(*Received August 27th, 1927.*)

In general albuminuria is accompanied by a decrease in the proportion of albumin in the blood, and the object of this investigation is to determine whether blood-albumin passes through the kidney unchanged or if there is a difference between blood- and urinary albumin.

It was shown in a recent paper [Hewitt, 1927] that proteins when purified possess a characteristic optical rotatory power and dispersion, and this provides a sensitive method for their identification. Hynd [1925] has examined albumins isolated from urine but the figures obtained for the specific rotatory powers of the specimens vary within very wide limits, probably due to non-removal of lipins and other substances which seriously affect the rotation.

Albumins excreted by nephritic patients have been isolated from urine, purified, and their rotatory power and dispersion determined.

Isolation and purification of urinary albumins.

Urine was collected in bottles containing toluene and worked up as soon after collection as possible. In some experiments the albuminous urines were first concentrated by ultrafiltration. For this purpose flat collodion discs were used, mounted with rubber washers in a large Seitz filter. The membranes were prepared by pouring a filtered 6 % solution of pyroxylin in a mixture of alcohol (50 vols.), ether (50 vols.) and acetic acid (10 vols.) on a flat glass plate. After leaving for a few minutes for the ether to evaporate, the glass plate was plunged into cold water, when the membrane became readily detachable. After washing thoroughly in water, the membrane was cut to shape and fixed in the Seitz filter. If the membranes were allowed to dry, they shrank and became impermeable. Experiments with samples of cellophane kindly supplied by the British Cellophane Company were not successful, filtration being much too slow. Negative pressure was applied to the filtrate side of the apparatus and a positive pressure up to 10 atmospheres was applied to the other side using a reservoir constructed by Mr Macarthy in this Hospital.

To the filtered (or concentrated) urine saturated ammonium sulphate solution was added to make the mixture at least 60 % saturated with ammonium sulphate and acetic acid was added until the reaction was about p_H 4·7. After allowing to stand the precipitated proteins were filtered off on a Büchner

funnel, dissolved in a small quantity of water, the solution neutralised, and cooled to 0° and then added drop by drop to about 8 times its volume of a mixture of alcohol (7 parts) and ether (3 parts) kept at − 15°. After standing at low temperature the precipitated proteins were filtered on a chilled Büchner funnel in the ice-chest, thoroughly washed with cooled alcohol-ether mixture, and with cold ether, and then rapidly transferred to a Soxhlet thimble and extracted for 20 hours with ether, metallic sodium being placed in the extraction flask to prevent any alcohol present from vaporising. After the first two hours' extraction, the ether and sodium were renewed. The extracted proteins were transferred to a vacuum desiccator and dried *in vacuo* over sulphuric acid for 48 hours, and then allowed to remain exposed to laboratory air for 24 hours, when the powdery solid was no longer readily electrified and was more easily "wetted," and hence dissolved in water more rapidly. The solid was pulverised, dissolved in water, any insoluble material (*e.g.* uric acid) filtered off or removed by centrifuging, and the solution made half saturated with ammonium sulphate by the addition of saturated ammonium sulphate solution. After allowing to stand overnight the precipitated globulins were filtered off, and the albumins precipitated in the mother-liquor by adding a further quantity of saturated ammonium sulphate solution and adjusting the reaction to about p_H 4·8 by adding 10 % acetic acid. After allowing to remain overnight the albumin was filtered off, dissolved in water, the solution neutralised and the above precipitation repeated. After one further repetition of the process the albumin was dissolved in a small quantity of distilled water, the solution placed in a collodion sac and dialysed against distilled water which was renewed frequently for at least 10 days. The clear dialysed solution was used for optical work.

Optical rotatory power and dispersion of urinary albumins.

The optical rotations of the solutions of the albumins obtained as described were determined in 1 dm. tubes, a Schmidt and Haentzsch polarimeter being used. The amount of protein present in the solutions was determined by Dr J. R. Marrack using the Kjeldahl method. In the following table specific rotatory powers for the mercury green line ($\lambda = 5461$ Å.) are quoted.

Patient	$[\alpha]_{5461}$	Diagnosis
1	− 76·9°	Chronic nephritis with oedema
2 *a*	− 78·9	Chronic nephritis with oedema
2 *b*	− 76·9	
2 *c*	− 79·6	
3	− 78·0	Subacute chronic nephritis, oedema and uraemia
4	− 74·8	Chronic nephritis, oedema and uraemia
5	− 77·5	Albuminuria of pregnancy; chronic nephritis
6	− 77·3	Albuminuria of pregnancy
Urinary albumin (mean)	− 77·6	
Serum-albumin [Hewitt, 1927]	− 78·1	

It will be seen that the mean values for urinary albumin (− 77·6°) and serum albumin (− 78·1°) are, within the limits of error, identical, so that it

is probable that urinary and serum-albumins are the same protein. The specific rotatory powers of the albumins were determined also for light of different wave lengths. The results were

Albumin	$\lambda=6660$	5780	5461	4358 Å.
Urinary (mean of 8)	$-47{\cdot}7°$	$-67{\cdot}3°$	$-77{\cdot}6°$	$-148{\cdot}1°$
Serum	$-47{\cdot}3°$	$-67{\cdot}0°$	$-78{\cdot}1°$	$-151{\cdot}5°$

The rather low figure $[\alpha]_{4358} - 148{\cdot}1°$ for urinary albumin can be explained, since some of the specimens were somewhat dark in colour and absorbed a considerable amount of blue light, rendering accurate measurements impossible. Apart from this, very close agreement is observed. It is therefore rendered extremely probable that serum-albumin passes through the kidney unchanged.

SUMMARY.

The albumins obtained from the urine of patients with chronic nephritis and albuminuria of pregnancy possess the same optical rotatory power and dispersion as serum-albumin, and are therefore probably identical with it.

The author's thanks are due to Dr J. R. Marrack for his helpful interest in this work, which was carried out under the auspices of the Freedom Research Fund.

REFERENCES.

Hewitt (1927). *Biochem. J.* **21**, 216.
Hynd (1925). *Lancet*, ii, 910.

CL. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

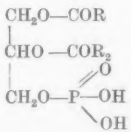
IV. FURTHER OBSERVATIONS ON DIGLYCERIDEPHOSPHORIC ACID.

By HAROLD JOHN CHANNON
and ALBERT CHARLES CHIBNALL.

From the Department of Experimental Pathology and Cancer Research, University of Leeds, and the Department of Physiology and Biochemistry, University College, London.

(*Received August 29th, 1927.*)

In a previous paper [Chibnall and Channon, 1927] it was shown that the ether extract of cabbage leaf cytoplasm contains very little, if any, phospholipin, in the commonly accepted use of this term. The major part of the ether-soluble phosphorus was present as the calcium salt of a diglyceridephosphoric acid of the type



Sufficient evidence was adduced to substantiate this structural formula, but the small amount of material then at our disposal did not enable us to obtain all the information which we considered was necessary for establishing the relationship of this acid to the two commoner phospholipins, lecithin and kephalin.

During the present summer we have prepared nearly 60 g. of the crude calcium salt from about 220 kg. of cabbage leaves, and have been able to investigate further two of the questions that had perforce been left open.

(1) In all our earlier work on this diglyceridephosphoric acid we had been troubled by the persistence with which small amounts of iron and nitrogen clung to what were considered purified products. We have now prepared this acid free from iron by dissolving it in a small volume of cold absolute alcohol. There remains undissolved a substance containing 8·9 % of iron and no nitrogen, which is extremely soluble in ether and is completely precipitated from ethereal solution by the addition of only one volume of alcohol. This explains why our previous efforts to purify the diglyceridephosphoric acid

by precipitation of its lead salt from ethereal solution by alcohol were unsuccessful. The yield of this unknown iron-containing substance is extremely small, 1·1 g. from 220 kg. of leaves. In view of the known connection in chlorotic leaves between chlorophyll synthesis and iron, we are undertaking a further investigation of this substance.

Although we have thus prepared the diglyceridephosphoric acid free from iron, we are still unable to remove the small amount of nitrogen (0·11 %). As to the nature of the substance giving rise to this nitrogen, we have no information. The natural suggestion would be that lecithin or kephalin was responsible for it. From the method of preparation, it seems improbable that the nitrogen was due to the presence of lecithin. In the preparation of the diglyceridephosphoric acid, the material precipitated from ether by acetone was repeatedly extracted with boiling acetone to remove certain other substances, the nature of which will be subsequently discussed. This treatment would tend to remove lecithin in the small amounts in which it could be present, for lecithin has a definite solubility in that solvent. Further, in this and previous work, we have purified the diglyceridephosphoric acid by repeated precipitation of its lead salt from ether solution by alcohol. This treatment would certainly have removed lecithin, and it seems probable that the small amount of kephalin which would be necessary to give rise to this percentage of nitrogen would have been removed also. The diglyceridephosphoric acid is also very soluble in cold acetone. In addition, as was previously reported, we have been unable to obtain at any time a precipitate with alcoholic cadmium chloride, and the nitrogen after the baryta hydrolysis in the preparation of the barium glycerophosphate remains insoluble in water. Hence we have no evidence whatever in favour of this 0·1 % of nitrogen being due to the presence of lecithin or kephalin, and it seems probable that there is some other ether-soluble nitrogen-containing substance present. This question must remain open until material is available in greater bulk.

(2) The nature of the fatty acids resulting from hydrolysis of the diglyceridephosphoric acid required more detailed investigation. We have now been able to show that the saturated acids are probably stearic and palmitic and that the unsaturated acids are linolic and linolenic. Oleic acid has not been identified, though that acid may be present in small amounts. A distinguishing feature is the low proportion of saturated acids. Levene, in his papers on lecithins and kephalins of animal origin, has shown that the acids consist of equal molecular proportions of saturated and unsaturated acids. But his more recent work on soya bean phosphatides has shown that this relationship may not hold in the plant, for his soya bean lecithin contained a low proportion of saturated acids. This is likewise the case with the diglyceridephosphoric acid. In the first place, the high degree of unsaturation of the mixed acids (iodine value, 143) is greater than that found in the acids from liver lecithins, which are characterised by the presence of arachidonic acid, which has four double bonds. We have found no trace of an acid more un-

saturated than linolenic in the mixed acids under discussion. In the second place, the fractionation of 13·2 g. of the mixed acids by means of the lead soaps yielded 2·85 g. of solid acids of iodine value 44, and 9·35 g. of liquid acids of iodine value 173. The major part of the latter fraction must have been linolic acid, for only 2·1 g. of hexabromostearic acid, equivalent to 0·8 g. of linolenic acid, were subsequently isolated from it. Therefore, the amount of saturated acids remaining in the liquid acids must have been small, and there is no doubt that the proportion of saturated acids in the original mixed acids was considerably less than one half.

The further evidence submitted in the present paper confirms our original contention that the free acid has the structural formula already given. It is therefore necessary to consider in some detail the class of substances to which this free acid and its salts are to be assigned. The free acid is an oil which is freely soluble in organic solvents, but has a very low solubility in water. The sodium salt is insoluble in ether, has a low solubility in cold alcohol and is soluble in water. The barium, calcium and lead salts are plastic solids which are insoluble in water, very readily soluble in ether, and are precipitated from ethereal solution by both acetone and alcohol. These properties, together with the fact that the acid and its salts readily darken on exposure to air, are essentially those of kephalin.

It is to be remembered that Thudichum divided the substances which give rise on hydrolysis to fatty acids, nitrogen and phosphorus, into two classes, the phosphatides and the cerebrosides. Leathes [1910] put forward reasons for altering these terms to phospholipin and galactolipin respectively. Thudichum's classification [1884] was based primarily on whether or not the substance contained phosphorus. His cerebrosides were nitrogen-containing but phosphorus-free fatty substances, while his phosphatides, as their generic name indicates, contained phosphorus but did not necessarily contain nitrogen. Thus, although his kephalophosphoric acid, the existence of which has yet to be proved, was nitrogen-free, it was classed as a phosphatide.

In his discussion of Leathes' nomenclature, MacLean [1918] defines the term lipins as "substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids, and containing in their molecule either nitrogen or nitrogen and phosphorus." The essential point of the classification of Leathes is thus the nitrogen content of the substance, and from this point of view it seems to us the less satisfactory. Since Thudichum's classification is based on the phosphorus content of the fat, *i.e.* phosphatides are "phosphorised" fats, whether they contain nitrogen or not, the substance discussed in this paper readily finds a place in it, while it remains outside that of Leathes. There is the further point that the terms phosphatide and cerebroside have been in international use since 1880 and are in use to-day. They are wider terms and can be used to embrace a greater number of similar substances than those suggested by Leathes, and until research has further elucidated the difficult question as to what further compounds of these types exist, it

seems to us preferable to keep to Thudichum's original definitions. If this be done the diglyceridephosphoric acid and its salts are phosphatides. Since the acid is chemically the parent acid of the two more commonly occurring phosphatides lecithin and kephalin, we propose to call it *phosphatidic acid* and the calcium salt found by us in the plant becomes calcium phosphatidate.

As the latter may thus be regarded as the simplest of the phosphatides so far reported, the question arises as to whether it may not be the precursor of lecithin and kephalin in the animal body. It is to be remembered that kephalin preparations have been reported to be contaminated by calcium. Thus Diaconow [1867] reported that the kephalin fraction (ether-soluble, alcohol-insoluble) from eggs contained calcium which can be removed by shaking its ethereal solution with HCl, and that the acid solution contained no phosphoric acid, showing that the calcium was not present in inorganic combination. Thudichum reported that after treatment of an ethereal solution of kephalin with HCl, the latter on evaporation yielded an ash containing calcium and iron. Parnas [1909] made similar observations and stated that an ethereal solution of crude kephalin loses its fluorescence when it is shaken with HCl. This fluorescence returns if the calcium-free ether solution is shaken with lime water. There would thus seem to be sufficient evidence that calcium in chemical combination with organic material in a form which is soluble in ether does exist in those tissues from which phosphatide preparations are usually made. This ether-soluble calcium appears in the kephalin fraction and can be removed by simple shaking with dilute mineral acid. In other words, it has similar solubility properties and is acted on by mineral acid in the same way as calcium phosphatidate. Further, Levene and Komatsu [1919], in their studies on cuorin, found that by careful fractionation of the phosphatide preparation from heart muscle they were able to obtain fractions which were beginning to approximate to various simpler kephalin derivatives, such as a monoglyceridephosphoric acid, etc. These may be decomposition products and this view is the one usually adopted; but there seems to us no sufficient reason why we should expect to find only pure kephalin and lecithin in such a tissue as, say, heart muscle. Because these phosphatides are extremely difficult to prepare in an analytically pure state, and because of the great ease with which they are oxidised, the conception has arisen that every so-called impurity in such preparations is the result of decomposition of the material through chemical manipulation or enzyme action. May not the alternative view be the more probable, namely, that these simpler products are normally present to some extent in such tissues, and that their presence is the cause of the variation in the N : P ratios on which the existence of so many phosphatides has been postulated, only to be overthrown on more careful examination?

Work is in progress with a view to obtaining further information as to the possible occurrence of nitrogen-free phosphatidic substances in animal tissues. Certain of the difficulties which will arise are obvious, not the least

of them being the necessity of ensuring that any such substance isolated has not arisen through the decomposition of the primary substances present. Recently a paper has appeared [Wadsworth, Maltaner and Maltaner, 1927], in which the authors claim to have obtained a nitrogen-free phosphatidic substance from heart muscle, after previous treatment of an extract with ammonia and calcium chloride. To this substance they attribute the structure of calcium phosphatidate. The chemical evidence which these authors have so far produced is wholly unsatisfactory. We shall defer detailed criticism of this paper until the results of our investigations on heart muscle, which are among the tissues which we have been studying for some time from this point of view, are more complete.

In discussing the analytical data which follow, we have continued to use the figures for distearylglycerophosphoric acid as a basis for theoretical calculation.

Experimental.

Preparation of the crude calcium salt.

220 kg. leaves from winter-sown cabbage picked in May were treated as described in the earlier paper. 220 g. ether-soluble substances were extracted, from which 58 g. crude calcium salt were obtained after precipitation with acetone and extraction of the insoluble material with hot acetone.

Crude calcium salt R.

0·1008 g. substance: 0·2077 g. CO_2, 0·0763 g. H_2O, 0·0222 g. ash.
0·2903 g. substance: 0·0043 g. Fe_2O_3 and 0·0506 g. $Mg_2P_2O_7$.
0·2902 g. substance: 0·0628 g. $CaSO_4$.
0·2903 g. substance: 0·0632 g. AgI.
0·1160 g. substance required (micro-Kjeldahl) 2·0 cc. 0·01 *N* acid.

	C	H	Ca	P	Fe	Ash	Glycerol	N
Found	56·19	8·40	6·37	4·86	1·00	22·03	8·54	0·24 %

2·90 g. on saponification yielded 1·77 g. of fatty acids or 61·1 % and 0·0512 g. of unsaponifiable matter or 1·8 %.

Isolation of phosphatidic acid.

50 g. crude calcium salt in 800 cc. ether were shaken once with 400 cc. *N*/2 H_2SO_4 and four times successively with 200 cc. *N*/4 H_2SO_4 to remove the calcium. The ether solution of the free acids was shaken four times with an aqueous solution of lead acetate, the ether solution of lead salts washed twice with water, and the solution concentrated to 300 cc. *in vacuo*. Two volumes of absolute alcohol were then added and the precipitated lead salts filtered off. These were dissolved in 200 cc. ether and reprecipitated with alcohol: yield, 36·0 g.

Lead salt of phosphatidic acid R.

0·0907 g. substance: 0·1675 g. CO_2, 0·0405 g. H_2O, 0·0288 g. ash.

	C	H	Ash
Found	50·36	7·41	31·75 %
Theory	51·47	8·14	32·30 %

When concentrated *in vacuo* the alcohol-ether mother-liquors gave a further precipitate of lead salt weighing 1·4 g., making a gross yield of 37·4 g. 34·9 g. of this lead salt in 900 cc. ether were shaken four times with *N*/4 HCl to remove the lead, washed four times with water and concentrated *in vacuo* to a syrup. 300 cc. cold absolute alcohol were then added to dissolve the phosphatidic acid. The insoluble material was removed by centrifuging, dissolved in ether and again shaken with *N*/4 HCl as before. A small amount of lead as chloride was removed, and, after washing, the ethereal solution was concentrated to 20 cc. *in vacuo*. Two volumes of alcohol were added with stirring, when the precipitated material collected as a brown gummy mass around the stirring rod. The yield of this insoluble material, which is the iron-containing material referred to in the introduction, was 1·1 g. The alcoholic solution on evaporation yielded 24·7 g. of phosphatidic acid. It is a pale olive-brown oil which flows slowly at room temperature, and clings tenaciously to traces of organic solvents. For analysis it was dried at 100° *in vacuo* for one hour, a treatment which does not affect its solubility in ether.

Phosphatidic acid R.

0·0771 g. substance: 0·1795 g. CO_2, 0·0663 g. H_2O.

0·2346 g. substance: 0·0374 g. $Mg_2P_2O_7$.

0·2306 g. substance (Zeisel-Fanto): 0·0728 g. AgI.

0·0938 g. substance required (micro-Kjeldahl) 0·80 cc. 0·01 *N* acid.

0·4692 g. substance required for neutralisation 13·4 cc. 0·1 *N* NaOH.

0·0938 g. substance absorbed 0·1079 g. iodine (Wijs); iodine value 115.

	C	H	P	M.W. (dibasic acid)	Glycerol	N
Found	65·71	10·09	4·42	700	12·5	0·11 %
Theory	66·57	10·94	4·40	704	13·1	0·0 %

3·51 g. on saponification with alcoholic potash gave 2·76 g. fatty acids of iodine value 143 and neutralisation value 199. Yield of fatty acids 78·6 %, theory for C_{18} saturated acid 80·6 %.

The total yield of phosphatidic acid represents 50 % and of the lead salt 55 % of the total phosphorus present in the original crude calcium salt. In view of the inevitable losses it is probable that about 60 % of the original phosphorus was present as calcium phosphatidate. The nature of the other 40 % of the phosphorus needs further investigation.

Products of hydrolysis of phosphatidic acid.

1. *Glycerophosphoric acid.* 15 g. phosphatidic acid dissolved in a small volume of alcohol were emulsified with water and added slowly to 750 cc.

boiling water in which had been dissolved 15 g. barium hydroxide. After standing on the water-bath for half an hour the precipitated barium soaps were filtered off, ground up with a further 300 cc. of baryta and boiled for one hour. This operation was repeated. Excess of barium was removed from the combined filtrates with carbon dioxide, and the filtrate from the barium carbonate concentrated to 50 cc. Two volumes of alcohol were added and the precipitated barium glycerophosphate filtered off. It was redissolved in water and boiled with charcoal until the filtrate was water-clear. The optical rotation was

$$[\alpha]^{20}_{\text{Hg green}} = +1{\cdot}3^\circ \ (l = 2,\ c = 3).$$

This rotation confirms our earlier finding, but, as we mentioned in the previous paper, we consider that Levene's lengthy lead treatment should be applied before the question of the true rotation of this substance can be answered. We have not yet accumulated sufficient material for this purpose.

2. *Saturated fatty acids.* The barium soaps from the above hydrolysis were converted into the free acids and saponified with alcoholic potash to make certain that all the phosphatidic acid had been decomposed. 10·7 g. fatty acids obtained in this way, together with 2·5 g. from a previous saponification, 13·2 g. in all, were converted into the lead soaps and extracted with ether. The ether-insoluble lead soaps yielded 2·85 g. solid acids, iodine value 44. From 2·47 g. of these acids 1·66 g. of crystalline fatty acids were prepared by fractionation from 95 % methyl alcohol. This material was further separated by means of absolute methyl alcohol into two fractions—*A*, weighing 0·80 g. and *B*, weighing 0·53 g. These analysed as follows:

A. 0·0702 g. substance: 0·1937 g. CO_2 and 0·0810 g. H_2O.
0·2855 g. substance required for neutralisation 10·55 cc. 0·1 *N* alcoholic NaOH.

B. 0·1039 g. substance: 0·2871 g. CO_2 and 0·1150 g. H_2O.
0·2351 g. substance required for neutralisation 3·47 cc. 0·25 *N* NaOH.

Calculated $C_{16}H_{32}O_2$: C, 74·92; H, 12·58; M.W. 256; M.P. 63–4°.
Calculated $C_{18}H_{36}O_2$: C, 75·93; H, 12·78; M.W. 284; M.P. 70–71°.
Found (*A*) C, 75·25; H, 12·82; M.W. 280; M.P. 57°.
(*B*) C, 75·37; H, 12·33; M.W. 271; M.P. 56°.

These results suggest that the saturated acids consist of about equal parts of stearic and palmitic acids.

3. *Unsaturated fatty acids.* The ether-soluble lead soaps yielded 9·35 g. of liquid acids, iodine value 173. An attempt was made to fractionate these acids as barium soaps by benzene containing 5 % of 95 % alcohol, but no insoluble fraction could be obtained. Prolonged centrifuging deposited a little slime which was not worth separating, showing that the original liquid acids contained a small residue of saturated acids and only a small amount, if any, of oleic acid. The free acids were regenerated from the barium soaps, and brominated in anhydrous ether at 0°. The ether-insoluble precipitate,

after well washing with ether to remove excess bromine, weighed 2·1 g. and analysed as follows:

0·1487 g. substance: 0·1582 g. CO_2 and 0·0560 g. H_2O.

0·0992 g. substance required (Stepanoff) 7·85 cc. $N/10$ thiocyanate.

Calculated $C_{18}H_{30}O_2Br_6$: C, 28·49; H, 3·99; Br, 63·26; M.P. = 180–1°.

Found C, 29·01; H, 4·18; Br, 63·23; M.P. = 180°.

The substance is hexabromostearic acid, showing the presence of linolenic acid in the original mixed fatty acids.

The brominated acids soluble in ether (12·7 g.) were concentrated *in vacuo* to a syrup and extracted with hot light petroleum. From the ether-light petroleum solution there were obtained by alternate concentration *in vacuo* and cooling crops of white crystalline material which together weighed 0·84 g. After recrystallisation from light petroleum, the material melted at 110–111°, and analysed as shown below. A sample recrystallised twice from ether melted at 113° and analysed as follows:

0·1058 g. substance: 0·1358 g. CO_2, 0·0560 g. H_2O.

0·1023 g. substance required (Stepanoff) 6·95 cc. $N/10$ thiocyanate.

Calculated $C_{18}H_{30}O_2Br_4$: C, 36·01; H, 5·38; Br, 53·28; M.P. 113–4°.

Found C, 35·80; H, 5·89; Br, 54·41; M.P. 113°.

The substance is tetrabromostearic acid, showing the presence of linolic acid in the original mixed fatty acids.

The ether-light petroleum mother-liquor contained 5·38 g. of acids containing 45·0 % Br, showing that a small amount of oleic acid may have been present in the original mixed fatty acids.

Summary.

Further observations on the calcium salt of a diglyceridephosphoric acid prepared from cabbage leaf cytoplasm have been made.

Reasons are given for considering the free acids and its salts as phosphatides.

The properties of the acid and its salts are discussed: since the acid is chemically the parent acid of lecithin and kephalin, the name phosphatidic acid has been assigned to it.

Phosphatidic acid differs from lecithin and kephalin, in which saturated and unsaturated fatty acids are present in equimolecular proportions, in that the greater part of the fatty acids present in the molecule is unsaturated.

The saturated acids are palmitic and stearic; the unsaturated acids are linolic and linolenic; oleic acid has not been identified; arachidonic acid is absent.

The possible occurrence of calcium phosphatidate in animal tissues is briefly discussed.

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REFERENCES.

Chibnall and Channon (1927). *Biochem. J.* **21**, 233.
Diaconow (1867). *Hoppe-Seyler's Med. Chem. Untersuch.* **2**, 221.
Leathes (1910). The fats (London).
Levene and Komatsu (1919). *J. Biol. Chem.* **39**, 91.
MacLean (1918). Lecithin and allied substances (London).
Parnas (1909). *Biochem. Z.* **22**, 411.
Thudichum (1884). A treatise on the constitution of the brain—discussed by MacLean in Lecithin and allied substances.
Wadsworth, Maltaner and Maltaner (1927). *Amer. J. Physiol.* **80**, 502.

CLI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VI.

By EDWARD HOYLE and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

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The experiments described in this communication are in continuation of a previously reported investigation on the behaviour of some of the inorganic constituents of lemon juice in the process of fractionation [Daubney and Zilva, 1926]. Nickel, cobalt, boron, iodine and phosphorus were considered in the first part of this research. The study of manganese and of iron in this connection became complicated by the presence of traces of these elements in the reagents used in the various manipulations. It was found that the mere absence of manganese and iron at the end of a blank experiment was not a fair indication that the two elements if found in the final fraction did not originate, at least in part, from the reagents used. Their presence in such fractions could only be considered of any significance if reagents free from all traces of the elements in question were used throughout the procedure. With this aim in view we have succeeded in preparing all our necessary reagents entirely free from interfering impurities. Manganese, iron, phosphorus and sulphur were investigated.

Manganese.

The scheme of fractionation adopted in this and in the following experiments was based on the improved method recently described by one of us [Zilva, 1927], eliminating the use of basic lead acetate. The method, although different in some details, did not differ essentially from the one employed by Daubney and Zilva in the first part of this research. The lemon juice was treated with excess of calcium carbonate, allowed to stand for about an hour and filtered. The filtrate was then concentrated by distillation in a vacuum at 50° to about a fifth of its original volume and treated with about half of its original volume of absolute alcohol. After filtering off the precipitate and removing the alcohol under reduced pressure at 50° the solution was diluted to the original volume of the juice with distilled water. After this the solution was fractionated according to the improved method mentioned above. All the reagents were previously purified.

Calcium carbonate was prepared from purified calcium chloride and sodium carbonate. The reagents were crystallised twice from distilled water. The resulting precipitate was thoroughly washed with distilled water and dried. To demonstrate that the chalk was free from manganese, 50 g. of it, *i.e.* about the amount required to neutralise 500 cc. of lemon juice, was stirred into an excess of dilute sulphuric acid. After filtering off the calcium sulphate the filtrate was concentrated to small bulk and any further precipitate of calcium sulphate removed. The liquid was then evaporated to dryness in a silica crucible and the residue tested for manganese by the method described later. Manganese was found to be totally absent. Ordinary precipitated chalk tested in the same way was found to contain appreciable quantities of manganese. The lead acetate was crystallised three times from dilute acetic acid, drained on a Büchner funnel and dried in the air. The absence of manganese from this reagent was demonstrated in the following ways.

(*a*) 10 g. of the salt (the quantity required for the treatment of an equivalent to 500 cc. of lemon juice) was dissolved in distilled water to which a drop of acetic acid was added to ensure perfect solution. The lead was then precipitated by the addition of an excess of sulphuric acid and the filtrate was evaporated to dryness in a silica crucible. Manganese was found to be absent from the residue.

(*b*) 10 g. of the lead acetate was dissolved as in (*a*) and magnesium sulphate added in slight excess above the quantity required to precipitate the lead. The mixture was then treated with two volumes of absolute alcohol, filtered, and the filtrate concentrated at 50° at low pressure, and eventually evaporated to dryness in a silica crucible. Manganese was absent from the residue.

No manganese was found in the filtrate when a solution of magnesium sulphate containing the quantity of the salt used in the manipulation was precipitated by the addition of two volumes of absolute alcohol, and the salt was therefore considered free from manganese.

The ammonia used in this experiment was also found to be free from all traces of manganese.

Bertrand's [1911] method, modified to suit our conditions, was employed in the estimation of manganese. In this method the manganese is oxidised to permanganate and is estimated colorimetrically. The ash of the various residues was heated with a few drops of sulphuric acid to drive off any chlorides present and after cooling was taken up in about 5 cc. of distilled water acidified with 1 cc. of concentrated nitric acid. The solution was then transferred to a specially selected test-tube suitable for colorimetric work, 5 drops of 10 % silver nitrate and about 0·1 g. of potassium persulphate were added and the mixture was boiled until the maximum pink coloration developed. Before matching, the solution was made up with distilled water to 10 cc.

A freshly made-up solution of potassium permanganate in graded dilutions and contained in suitable test-tubes was used as a standard. Manganous sulphate, which when oxidised as above yields equivalent quantities of per-

manganate, produces a solution which very often does not maintain its colour for any length of time and therefore the direct use of permanganate is more convenient.

It is possible in this way to determine manganese quantitatively within the limits of 0·005 and 0·15 mg., although the highest degree of accuracy is obtained within the range of 0·01–0·10 mg. in 10 cc. Below 0·005 mg. the coloration is perceptible but quantitative differentiation is unreliable. Above 0·10 mg. the difference in colour also gradually becomes less marked and solutions very much above this concentration show a tendency not to retain the pink colour and to deposit manganese dioxide. Although the latter compound can be reoxidised the procedure is troublesome. The sample of nitric acid used by us showed traces of manganese, but as this was the only reagent contaminated with this element, blanks, containing the quantity of nitric acid used in the entire determination including the incineration, were employed. It is to be pointed out that in the oxidation of manganese salts with nitric acid and persulphate the use of too much or too little of the acid influences the stability of the pink coloration.

Five batches of lemon juice were fractionated, two of 250 cc. each and three of 500 cc. each. In none of these could any manganese be detected in the final fraction. The decitrated lemon juice, and the supernatant liquid from which the active fraction was precipitated, contained 0·01 mg. of manganese per 500 cc. of juice.

Table I. *Mg. of Mn in fractions from 250 cc. of tomato juice.*

	No addition of Mn			0·02 mg. Mn as $MnSO_4$ added to supernatant liquid from neutral lead acetate ppt.	
No. of batch	Supernatant liquid from neutral lead acetate ppt.	Supernatant liquid from basic ppt.	Basic precipitate	Supernatant liquid from neutral lead acetate ppt.	Basic precipitate
I	0·055	0·060	nil	0·080	nil
II	0·040	0·040	—	0·060	,,
III	0·080	0·080	nil	0·090	,,
IV	0·060	0·060	,,	0·070	,,
V	0·060	0·060	,,	0·080	,,
VI	0·060	0·055	,,	0·075	,,

Apart from the above experiments a preliminary investigation on tomato juice produced similarly negative results. This work was done before the new method of fractionation was introduced and consequently the fractionation was carried out by the basic lead acetate method. The latter reagent was obtained free from manganese by recrystallising a sample of ordinary basic lead acetate from a saturated boiling water solution. The non-active material was first removed from the tomato juice by precipitation with two volumes of concentrated alcohol followed after removal of the alcohol from the filtrate by treatment with purified neutral lead acetate. The fraction precipitated by the basic lead acetate was taken up in dilute acetic acid and decomposed

with sulphuric acid. On removing the lead sulphate, the filtrate was evaporated, incinerated and tested for manganese as previously described.

In this experiment the batches were divided into two series, to one of which $MnSO_4$ equivalent to 0·02 mg. of Mn per 250 cc. was added to the supernatant liquid from the neutral lead precipitate, in order to see whether inorganic manganese would be precipitated under these conditions. As will be seen from Table I no manganese was found in any of the final fractions. Similar fractions from tomato juice (decomposed however with $MgSO_4$) were tested biologically on guinea-pigs and were found to be active.

Iron.

The reagents used in the production of the active fractions for the estimation of iron were purified as in the case of the manganese estimations.

The calcium chloride and the sodium carbonate used in the preparation of the calcium carbonate were found free from iron after several crystallisations. Similarly the recrystallised neutral lead acetate on being decomposed with sulphuric acid was found to contain no iron. Nor did the ammonia and magnesium sulphate show the presence of any iron. The nitric acid contained traces of iron. This was compensated for by using blanks in the estimations. The iron was determined colorimetrically as ferric ferrocyanide, ferric alum stabilised by the addition of a few drops of hydrochloric acid being used for the standards. To the solution contained in specially selected test-tubes, 1 cc. of 0·5 % potassium ferrocyanide and a drop of concentrated hydrochloric acid were added for the production of the Prussian blue and the contents brought up to 10 cc. The optimum range was found to be between 0·02 and 0·15 mg. per 10 cc. By observing these conditions a colour of uniform tint and requisite durability was obtained. The various fractions were prepared, incinerated as for the manganese estimations and taken up in hydrochloric acid. Since about 0·15–0·26 mg. of iron per equivalent of a litre of original juice was found to be present, it was thought of interest to study the behaviour of this residual iron on dialysis through collodion membranes soaked in 88 % and in 95 % alcohol respectively [Zilva and Miura, 1921; Connell and Zilva, 1924; Daubney and Zilva, 1926]. This was carried out in the way described by

Table II. *Mg. of Fe in undialysed and dialysed fractions. Calculated for 1 litre of lemon juice.*

No. of batch	Decitrated alcohol-treated lemon juice	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
			88 %	95 %
I	2·4	0·16	0·16	0·12
II	1·0	0·20	0·08	nil
III	1·6	0·28	0·14	,,
IV	0·6	0·20	0·04	,,
V	1·0	0·16	0·16	,,
VI	1·3	0·16	0·12	,,
VII	—	0·24	0·16	,,

Daubney and Zilva. Iron was estimated in 50 cc. of decitrated juice and in equivalents of 250 cc. of the other fractions which as before were all reduced to 10 cc. for the estimation. Table II gives a summary of the results calculated for an equivalent of 1 litre of juice.

It is seen that the iron passed through an 88 % thimble only to a small extent whilst, with the exception of one case, the 95 % thimbles permitted the entire passage of the iron. This will be discussed in a later section of the paper.

Phosphorus.

The reagents used in the fractionation were purified as before. The calcium carbonate was tested in the following ways. (*a*) The requisite quantity of chalk was treated with sulphuric acid until slightly acid, filtered, and the filtrate concentrated to about 5 cc. After filtering off any further calcium sulphate which separated out during concentration, the solution was tested for total phosphorus by the Martland and Robison modification [1926] of the Briggs method. (*b*) In this case the calcium carbonate was decomposed with citric acid using a quantity of the acid which was approximately equivalent to that contained in 100 cc. of lemon juice. After neutralising the acid it was treated with two volumes of alcohol, filtered, and the alcohol removed by distillation from the filtrate which was concentrated to about 5 cc. and tested for phosphorus by the above method. No traces of phosphorus could be detected by either method. Ordinary samples of precipitated chalk treated as above showed a content of 0·1 mg. of phosphorus per 10 g. of calcium carbonate.

The neutral lead acetate was tested for phosphorus after being decomposed by sulphuretted hydrogen and was found to be free, as was also the ammonia.

Lemon juice was fractionated as before, except that the final fraction was decomposed by sulphuretted hydrogen instead of by magnesium sulphate. Fractions treated in this way, as previously pointed out by one of us, lose their activity. It was found, however, that the magnesium sulphate treatment owing to the almost unavoidable presence of small quantities of this salt in the final fraction, made the ignitions of the organic matter, as carried out in the above method, troublesome. This inactivation of the fraction would not of course interfere with the inorganic content of the fraction. The analysis of six batches of the pure fraction equivalent to 100 cc. yielded 0·12, 0·13, 0·13, 0·14, 0·12, 0·11 mg. respectively and, as will be seen from Table III, the phosphorus in the final fractions dialysed only to a very small extent through collodion thimbles of 88 % alcohol index, whilst the thimbles with a 95 % index permitted the entire phosphorus to diffuse in the course of three days.

Attempts to estimate how much of the phosphorus was inorganic failed, owing to the turbidity and to the modification in the tint obtained by adding the Briggs reagent before ignition.

It may however be noted that disodium hydrogen phosphate and potassium

dihydrogen phosphate in equivalent dilutions dialysed in the same way as the phosphorus of the final fraction.

Table III. *Mg. of P in final fractions. Calculated for* 1 *litre of lemon juice.*

No. of batch	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
		88 %	95 %
I	1·28	1·13	nil
II	1·22	0·97	,,
III	1·37	0·96	,,
IV	1·17	0·95	,,
V	1·10	0·74	,,
VI	1·14	0·92	,,
VII	1·11	1·08	,,
VIII	1·20	1·00	,,

The figures for total phosphorus obtained in these experiments are rather lower than those previously obtained [Zilva, 1924; Daubney and Zilva, 1926], but this no doubt is due to the use of purified reagents.

Sulphur.

In spite of the use of reagents free from sulphur, quantities of this element were found in the final fraction which, considering the nature of the investigation, were of a high order. These figures were obtained without incinerating the preparation by direct precipitation as barium sulphate out of the solution which was previously heated with hydrochloric acid in order to convert any "ethereal" into inorganic sulphur. In nine batches, equivalent to 1 litre of original juice, 2·0, 3·6, nil, nil, 0·8, 2·3, 0·9 mg. were found. The sulphur in the active fraction diffused only partly through an "88 %" thimble, but entirely through a "95 %" collodion thimble. In view of these high and variable results the further effort of determining the total sulphur was not considered justified.

Conclusions.

In the two sections of this enquiry the active fraction from lemon juice has been examined for iron, phosphorus, sulphur, iodine, manganese, nickel, cobalt and boron. The last four elements were found to be absent and consequently, if at all associated with the antiscorbutic activity, they must be so in quantities not detectable by our methods. Of those present, iodine may also be excluded since, as has been shown by Daubney and Zilva, no evidence could be obtained that any of it diffused through a collodion thimble with a 95 % alcohol index. Antiscorbutic solutions dialysed under the same conditions lose their activity. On the other hand the iron, phosphorus and sulphur of the active fraction behaved on dialysis in the same way as the active principle. One must however be very cautious in postulating a connection between them and the antiscorbutic activity. These inorganic constituents cannot be responsible for the activity *per se* as they are almost ubiquitous in animal and plant tissues, whilst the distribution of the anti-

scorbutic factor in these tissues is limited to certain sources only. If there be any connection, these elements must be either in physical or chemical association with some other substance or radicle or themselves be in a definite physical state responsible for their physiological function. Needless to say this investigation offers no indication which would justify such hypotheses. The possibility that the presence of iron, phosphorus and sulphur in the active fraction is of significance must therefore await consideration until our knowledge of the problem is more advanced.

SUMMARY.

The concentrated antiscorbutic fraction from lemon juice does not contain any manganese but contains traces of iron, phosphorus and sulphur. These elements of the active fraction behave on dialysis like the active principle.

REFERENCES.

Bertrand (1911). *Bull. Soc. Chim.* **9**, 361.
Connell and Zilva (1924). *Biochem. J.* **18**, 641.
Daubney and Zilva (1926). *Biochem. J.* **20**, 1055.
Martland and Robison (1926). *Biochem. J.* **20**, 847.
Zilva (1927). *Biochem. J.* **21**, 354.
Zilva and Miura (1921). *Biochem. J.* **15**, 422.

CLII. LIVER DIASTASE.

By E. F. LESSER.

From the Laboratorium der städt. Krankenanstalten, Mannheim.

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In his paper on liver diastase, Eadie [1927] says, "Lesser...has advocated another view, viz. that the glycogen and amylase are, as it were, 'locked up' in different compartments of the cell. This explanation unfortunately adds little to our knowledge because it suggests no mechanism whereby the two are kept separate."

Unfortunately Eadie quotes my researches only to the year 1921. In 1923 I showed [Lesser and Zipf, 1923] that homologous alcohols in isocapillary concentrations increase sugar formation in the perfused frog's liver by approximately the same amount, and later [Lesser, 1925] that this effect occurs under reversible conditions. Finally it has been shown [Lesser, 1926] that the effect is also similar in a perfusion fluid buffered with carbon dioxide and bicarbonate at p_H 7·4 and 7·6.

On the basis of these findings I have concluded, in agreement with Warburg [1923], that a boundary surface-phenomenon is involved, and have assumed that approximately $\frac{2}{3}$ of the liver diastase is adsorbed on the surfaces of the cell and is therefore unable to act on the liver glycogen. Similar experiments with the isolated parotid gland of the mouse [Lesser, 1927] have led to precisely the same results. Eadie is therefore incorrect in stating that I have not suggested a mechanism whereby the diastase and glycogen are kept separate.

REFERENCES.

Eadie (1927). *Biochem. J.* **21**, 320.
Lesser (1925). *Biochem. Z.* **156**, 161.
—— (1926). *Biochem. Z.* **171**, 83.
—— (1927). *Biochem. Z.* **184**, 125.
Lesser and Zipf (1923). *Biochem. Z.* **140**, 439.
Warburg (1923). *Jahresber. ges. Physiol.* **1**, 136.

CLIII. THE EFFECT OF HYDROGEN ION CONCENTRATION AND PROTEIN CONCENTRATION ON THE OSMOTIC PRESSURE OF SERUM-PROTEINS.

By JOHN MARRACK and LESLIE FRANK HEWITT.

From the Hale Clinical Laboratory, London Hospital, E. 1.

(*Received August 16th, 1927.*)

Several workers have shown that the osmotic pressure of serum- or plasma-proteins, in solutions of increasing protein concentration, increases more rapidly than the protein concentration.

Verney [1926] has shown that this relation is such as would be found if the protein in solution occupied many times its dry volume. There are, however, other factors which will cause a deviation from a linear relation between observed osmotic pressure and protein concentration. Particularly, there is an excess of diffusible ions in the protein solution over that in the outside solution, due to the Donnan effect; this excess of diffusible ions would, if the solutions were ideal, have an osmotic pressure, which we will call the Donnan pressure, over and above that due to the protein itself; and this Donnan pressure would be proportional to the square of the protein concentration, when the salt concentration of the outside fluid and the reaction were fixed. As a consequence the observed osmotic pressure (y) and protein concentration (x), at any particular p_H, would be connected by an equation of the form

$$y = ax + bx^2 \qquad \text{.....(1),}$$

a parabola convex to the axis of x, very similar to the curves found by Mayrs [1926] and Verney [1926].

The term b in equation (1) would vary with the p_H, so that the observed osmotic pressure should also vary with the p_H. Mayrs however was unable to detect such variation.

We have therefore studied the effect of varying protein concentration and p_H on the observed osmotic pressure, with the idea that although the actual effect of the Donnan equilibrium would not agree with that calculated for ideal solutions, it would still be demonstrable, and with the hope of showing deviations from ideality which will affect the relation between the observed osmotic pressure and the protein concentration.

The excess of diffusible ions in the protein solution, and the osmotic pressure resulting therefrom.

Symbols.

[Pr] = concentration of protein in the protein solution in g. per 1000 g. H_2O.

[Pr^-] = concentration of protein ions in the protein solution in millequivalents per 1000 g. H_2O.

[BPr] = concentration of base bound by protein in the protein solution in millequivalents per 1000 g. H_2O.

[A_P^-] = concentration of diffusible anions in the protein solution in millequivalents per 1000 g. H_2O.

[B_P^+] = concentration of diffusible kations in the protein solution in millequivalents per 1000 g. H_2O.

[B_P] = concentration of total base in the protein solution in millequivalents per 1000 g. H_2O.

[Cl_P] = concentration of chlorine in the protein solution in millequivalents per 1000 g. H_2O.

[Cl_P^-] = concentration of chlorine ions in the protein solution in millequivalents per 1000 g. H_2O.

[HCO_{3P}^-] = concentration of bicarbonate ions in the protein solution in millequivalents per 1000 g. H_2O.

[A_E^-] = concentration of diffusible anions in the external solution in millequivalents per 1000 g. H_2O.

[B_E^-] = concentration of diffusible kations in the external solution in milliequivalents per 1000 g. H_2O.

a_{A_P}, a_{B_P}, a_{Cl_P}, etc. = the activities of the ions in the protein solution.

a_{A_E}, a_{B_E}, a_{Cl_E}, etc. = the activities of the ions in the external solution.

$f_{A_P}, f_{B_P}, f_{Cl_P}$, etc. and $f_{A_E}, f_{B_E}, f_{Cl_E}$, etc. = the activity coefficients of the ions in the two solutions.

Then $$a_{A_P} = f_{A_P}[A_P^-];\ a_{A_E} = f_{A_E}[A_E^-], \text{ etc.} \qquad \text{.....(2).}$$

Let $$[Pr^-] = k\,[BPr].$$

Now $$[B_P^+] = [A_P^-] + [Pr^-] = [A_P^-] + k\,[BPr] \qquad \text{.....(3),}$$

and

$$f_{A_P}[A_P^-].f_{B_P}[B_P^+] = a_{A_P}.a_{B_P} = a_{A_E}.a_{B_E} = f_{A_E}[A_E^-].f_{B_E}[B_E^+] = f_{A_E}.f_{B_E}.[A_E^-]^2 \qquad \text{.....(4).}$$

$$\therefore\ f_{A_P}.f_{B_P}.[A_P^-]\{[A_P^-] + k\,[BPr]\} = f_{A_E}.f_{B_E}.[A_E^-]^2.$$

$$\therefore\ [A_P^-] = \left[\frac{f_{A_E}.f_{B_E}}{f_{A_P}.f_{B_P}}[A_E^-]^2 + \frac{k^2}{4}[BPr]^2\right]^{\frac{1}{2}} - \frac{k}{2}[BPr]$$

$$= \left(\frac{f_{A_E}.f_{B_E}}{f_{A_P}.f_{B_P}}\right)^{\frac{1}{2}}[A_E^-] + \frac{k^2}{8}\left(\frac{f_{A_P}.f_{B_P}}{f_{A_E}.f_{B_E}}\right)^{\frac{1}{2}}.\frac{[BPr]^2}{[A_E^-]} - \frac{k}{2}[BPr] \qquad \text{.....(5).}$$

If k [BPr] is small compared with $[A_E^-]$;

similarly $$[B_P^-] = \left(\frac{f_{A_E} \cdot f_{B_E}}{f_{A_P} \cdot f_{B_P}}\right)^{\frac{1}{2}} [A_E^-] + \frac{k^2}{8}\left(\frac{f_{A_P} \cdot f_{B_P}}{f_{A_E} \cdot f_{B_E}}\right)^{\frac{1}{2}} \frac{[BPr]^2}{[A_E^-]} + \frac{k}{2}[BPr] \quad ...(6).$$

$$\therefore [A_P^-] + [B_P^-] = 2\left(\frac{f_{A_E} \cdot f_{B_E}}{f_{A_P} \cdot f_{B_P}}\right)^{\frac{1}{2}} [A_E^-] + \frac{k^2}{4}\left(\frac{f_{A_P} \cdot f_{B_P}}{f_{A_E} \cdot f_{B_E}}\right)^{\frac{1}{2}} \frac{[BPr]^2}{[A_E^-]} \quad .....(7).$$

If the effect of the protein on the activity coefficients of the diffusible ions is negligible, this becomes

$$[A_P^-] + [B_P^-] = 2[A_E^-] + \frac{k^2}{4}\frac{[BPr]^2}{[A_E^-]},$$

that is, the excess of diffusible ions in the protein solution equals

$$\frac{k^2}{4} \cdot \frac{[BPr]^2}{[A_E^-]}.$$

The osmotic pressure at 37° due to this excess, when $[A_E^-] = 160$, will be

$$26 \times 0{\cdot}938 \times \frac{k^2}{4}\frac{[BPr]^2}{[A_E^-]} \text{ cm. } H_2O.$$

The coefficient 0·938 is derived from Bronsted's [1921] formula

$$1 - \phi = az_1z_2\sqrt{\mu} + \beta c,$$

and the values for the activity coefficients at this ionic strength from Scatchard [1925].

If we assume that the activity coefficient of the protein does not change with varying protein concentration, the observed osmotic pressure (π) at constant p_H will be given by the equation

$$\pi = 26{,}000\frac{[Pr]}{M} + 26 \times 0{\cdot}938 \times \frac{k^2}{4} \times \frac{[BPr]^2}{160} \quad .....(9),$$

(where M is the molecular weight of the protein), which is a parabola convex to the [Pr] axis. Also, if the activity coefficient of the protein is not affected by change of hydrogen ion concentration, the observed osmotic pressure of a solution of constant protein concentration will rise with rising p_H by the amount,

$$\Delta\pi = \frac{26 \times 0{\cdot}938}{4 \times 160} \times k^2 \times 0{\cdot}097^2 [Pr]^2 [(p_{H_2} - 5{\cdot}26)^2 - (p_{H_1} - 5{\cdot}26)^2] \quad .....(10)$$

using equation (11) (see p. 1135) for [BPr].

If $k = 1$, the second term in equation (9) is equal to 13·1 cm. when [Pr] = 70 and the p_H is 8·0; and $\Delta\pi$ in equation (10) is equal to 7·6 cm. when [Pr] = 70 and $p_{H_1} = 7{\cdot}0$, $p_{H_2} = 8{\cdot}0$; thus the effect of this unequal distribution of diffusible ions will be by no means negligible if the assumptions with regard to activity coefficients are correct and the protein salt is fully dissociated.

Methods.

Horse serum was separated from blood obtained from the slaughter house and allowed to clot. Human serum was obtained by shaking blood from two normal males (J. M. and L. H.) with glass beads to remove fibrin and centrifuging. The human sera were obtained under sterile conditions and both horse and human sera were filtered through a Seitz filter before use. The lower concentrations of protein were obtained by dilution with 0·9 % sodium chloride solution.

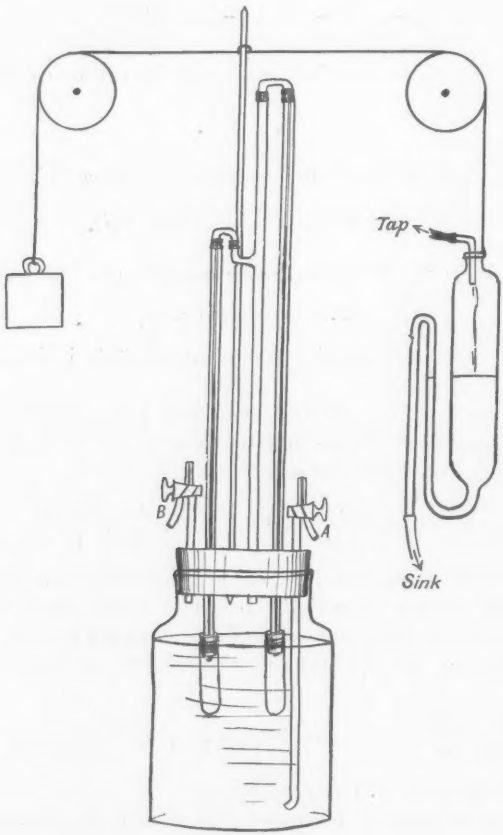

Fig. 1.

The osmotic pressures were measured with the apparatus shown in Fig. 1. The large bottle contained about 1 l. of a bicarbonate-carbon dioxide buffer mixture, containing phenolsulphonephthalein. The bicarbonate solution was first sterilised by boiling in a litre flask; CO_2 was then passed into it through

a sterile cotton-wool filter, until the p_H was approximately that required. When necessary the p_H was adjusted more accurately after the solution had been placed in the bottle by running a CO_2-air mixture of known composition in through the tap *A* and out through *B*.

When the apparatus was in a water-bath the pressure inside the bottle was adjusted to atmospheric pressure by opening tap *B*.

Collodion sacs were made inside test-tubes using an 8 % solution of collodion (British Drug Houses) in a mixture of alcohol (50 volumes), ether (50 volumes) and glacial acetic acid (10 volumes). We are indebted to Mrs Dover who prepared many of the sacs used.

These sacs were attached to pressure tubing with rubber bands; they were made to hold about 8 cc. To check their impermeability to protein they were filled with serum, the rubber tubing was closed with a glass rod, and the sacs were placed for a night in normal saline, which was then tested with sulphosalicylic acid; if the faintest cloud appeared the sacs were rejected.

The sacs were found to be readily permeable to phenolsulphonephthalein; if they were filled with water and placed in a phenolsulphonephthalein solution over night, the concentrations of dye in the external and internal fluids were found to be the same next morning. The permeability was not altered by the process of sterilisation.

The capillary tubes had a bore of about 1 mm.; it was found that narrower tubes were unsatisfactory as the protein solution was liable to stick. Dilution of the protein solutions could be avoided by forcing the capillary tubes well into the collodion sacs so that the fluid rose in the capillary to about the height anticipated.

Those parts of the apparatus which could safely be heated were sterilised by autoclaving or by dry heat. The remainder, including the collodion membranes, were sterilised by soaking for 15 minutes with 70 % alcohol, which was washed off with sterile saline, or with alcohol and ether if the parts were required to be dry. The whole apparatus was assembled with aseptic precautions by an operator wearing sterile gloves.

These methods of sterilisation proved entirely satisfactory; even in subsequent experiments with egg-albumin we have had no bacterial contamination. The Seitz filters have proved most valuable when dealing with protein solutions which were not sterile to start with.

The apparatus was kept at 0° in a bath of ice and water for one day in order to let substances such as urea and electrolytes other than those present in the buffer solution dialyse out. The external fluid was then changed and the apparatus placed in a water-bath at 37° for 2 or more days. It was gently rocked and finally allowed to stand with the capillary tubes vertical; this final period without rocking was necessary, as during the short periods during which the bottle was tilted the vertical heights of the columns in the capillary tubes were reduced.

In the later experiments the gas pressure above the column of fluid was

equal to that above the external fluid; in order to read the osmotic pressures it was only necessary to mark the heights of the columns of fluid in the capillary tubes, remove the apparatus from the water-bath and read the levels of these marks and of the surface of the external fluid with a vertical mirror scale.

In the earlier experiments the apparatus was slightly different. Taps were carried on the ends of the capillary tubes to which the collodion sacs were attached. These taps were kept closed during the dialysis. When an osmotic pressure was to be read the height of the column of fluid in a capillary was marked, and without removing the apparatus from the water-bath the tap was connected with a water manometer and opened. The manometer pressure was then adjusted until the surface of the protein solution in the capillary was the same as before the tap was opened; this gave the gas pressure above the column in the capillary tube. The height of the mark on the capillary tube was then measured in the same way as in the later experiments; the osmotic pressure was equal to the pressure of this column of fluid plus the manometer pressure. This method was much less satisfactory than the simpler method of the later experiments and the results are given only to show that even with less accurate methods fairly consistent results can be obtained.

In calculating the osmotic pressure, allowance was made for the specific gravity of the protein solutions, and for the rise due to surface tension.

After the osmotic pressures had been read, the apparatus was replaced in the water-bath to reheat (little cooling took place during the short time required for reading the heights of the columns) and a sample of gas was taken into a gas sampler full of mercury attached to tap *B*; clean mercury was run in by tap *A* at the same time to drive the gas out. Before this process dead spaces were filled with mercury. This gas sample was analysed in a Haldane apparatus.

A sample of the external fluid was then taken and the protein solutions were run into test-tubes with precautions to avoid letting the fluids in the capillaries run out, as these fluids might differ from those in the sacs.

The *total nitrogen* in the internal fluids was estimated by Kjeldahl's method; since other nitrogenous substances would have diffused away into the large volume of external fluid this nitrogen was assumed to be that of protein and the protein present was calculated, using 6·45 as the factor.

Chlorides were estimated by heating 1 cc. of fluid in a water-bath with excess of silver nitrate and nitric acid, and titrating the excess of silver nitrate with potassium thiosulphate, using iron alum as indicator. The error in this estimation was not more than 0·5 %.

Bicarbonates were estimated in the external fluid by boiling 20 cc. with a slight excess of *N*/10 HCl and titrating the excess with *N*/10 NaOH with methyl red as indicator. The bicarbonate concentrations in the protein solutions were calculated on the assumption that the ratio

$$\frac{HCO_3 \text{ in protein solution}}{HCO_3 \text{ in external solution}} \text{ was the same as } \frac{Cl \text{ in protein solution}}{Cl \text{ in external solution}}.$$

The p_H of the external fluid was calculated from the CO_2 tension and bicarbonate concentration by the formula

$$p_H = 6{\cdot}1 + \log [BHCO_3^-] - \log pCO_2 - \log 0{\cdot}0326.$$

The *total base* was estimated by the method of Stadie and Ross [1925]. The proteins of the protein solutions were first precipitated with trichloroacetic acid. In the external fluids the total base was found to be equivalent to the sum of the chloride and bicarbonate found, within 1 % in almost all cases.

In the estimations of chlorides and total base all quantities under 5 cc. were weighed, in order to avoid errors in the discharge of pipettes.

Specific gravities were estimated by weighing in a pyknometer; dry weights by drying a known volume to constant weight in an oven at 100°–110° and cooling in a desiccator.

In preliminary experiments it was found that appreciable loss of CO_2 by diffusion through the rubber stopper, and changes in alkalinity of the buffer mixture from solution of glass, did not occur.

Base bound by protein.

The values for [BPr] given in Table I are obtained by subtracting [Cl] + [HCO_{3P}] from the total [B_P]. The estimation by this means must be inaccurate as it is given by the small difference between two large quantities; nevertheless the results are fairly consistent. For human serum-proteins the results and their relation to p_H (Fig. 2) agree fairly well with the formula given by Hastings, Salvesen, Sendroy and Van Slyke [1927]

$$[BPr] = 0{\cdot}097\ [Pr]\ (p_H - 5{\cdot}26) \text{ (curve A, Fig. 2)} \quad \ldots\ldots(11),$$

though they are slightly higher[1], but our results for horse serum-protein are consistently higher than those given by the formula of Van Slyke, Wu and MacLean [1923]

$$[BPr] = 0{\cdot}068\ [Pr]\ (p_H - 4{\cdot}80) \text{ (curve B, Fig. 2)} \quad \ldots\ldots(12).$$

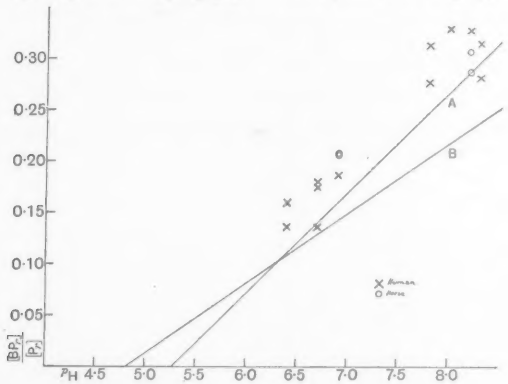

Fig. 2. Relation of base bound by protein to p_H.

[1] The p_H in our calculations is that of the external fluid, not of the protein solution; the effect of this however is very small and in the opposite direction to the difference found.

Table I. *Distribution of* Cl, HCO_3 *and base.*

		External fluid				Protein solution								
Expt. No.	Serum	p_H	[Cl]	$[HCO_3]$	[Cl]+ $[HCO_3]$	[Pr]	[Cl]	$[HCO_3]$	[B]	[BPr]	$\frac{[BPr]}{[Pr]}$	$\frac{[Cl_P]}{[Cl_E]}$	$\frac{[B_E]}{[B_P]}$	$\frac{[Cl_P][B_E]}{[Cl_E][B_P]}$
11 *b*	J. M.	6·4	142·0	—	—	57·2	142·3	—	—	—	—	1·002	—	—
5 *a*	„	6·7	139·9	14·5	154·4	63·9	138·7	14·4	161·8	8·7	0·136	0·991	0·9545	0·963
5 *b*	„	6·7	139·9	14·5	154·4	26·3	140·4	14·5	159·5	4·6	0·175	1·003	0·969	0·966
3 *a*	„	6·9	144·9	15·0	159·9	50·4	143·7	14·9	168·0	9·4	0·187	0·9915	0·952	0·961
3 *b*	„	6·9	144·9	15·0	159·9	34·5	145·0	15·0	—	—	—	1·001	—	—
12 *a*	„	7·8	142·9	14·8	157·7	50·3	140·1	14·5	170·3	15·7	0·314	0·980	0·9265	0·943
13 *a*	„	8·0	141·6	14·9	156·5	62·3	136·2	14·4	171·1	20·5	0·330	0·962	0·915	0·951
4 *a*	„	8·3	139·9	14·5	154·4	67·8	135·1	14·0	167·9	18·8	0·282	0·965	0·9195	0·948
4 *b*	„	8·3	139·9	14·5	154·4	37·1	137·1	14·2	163·2	11·9	0·315	0·987	0·946	0·965
6 *a*	„	6·4	288·9	14·6	303·5	66·9	292·2	14·8	316·1	9·1	0·136	1·011	0·960	0·949
6 *b*	„	6·4	288·9	14·6	303·5	36·7	292·3	14·8	—	—	—	1·021	—	—
7 *a*	„	8·2	288·2	14·6	302·8	64·8	283·1	14·3	318·6	21·2	0·328	0·982	0·950	0·969
7 *b*	„	8·2	288·2	14·6	302·8	32·2	287·0	14·5	—	—	—	0·996	—	—
11 *a*	L. H.	6·4	142·0	15·0	157·0	74·0	140·8	14·9	167·5	11·8	0·160	0·9915	0·9375	0·944
9 *a*	„	6·7	142·6	15·0	157·6	67·6	139·7	14·7	166·6	12·2	0·180	0·9795	0·946	0·955
9 *b*	„	6·7	142·6	15·0	157·6	40·9	141·5	14·9	—	—	—	0·992	—	—
10 *a*	„	7·8	142·4	14·9	157·3	63·6	137·1	14·4	—	—	—	0·963	—	—
12 *b*	„	7·8	142·9	14·8	157·7	68·6	138·2	14·3	171·5	19·0	0·277	0·967	0·920	0·949
1 *a*	Horse	6·9	125·4	30·6	156·0	70·5	122·0	29·7	166·4	14·7	0·208	0·971	0·9375	0·963
1 *b*	„	6·9	125·4	30·6	156·0	42·5	124·0	30·2	163·0	8·8	0·207	0·989	0·957	0·966
2 *a*	„	8·2	124·4	29·4	153·8	68·9	118·3	27·9	167·4	21·2	0·308	0·951	0·919	0·966
2 *b*	„	8·2	124·4	29·4	153·8	42·6	121·6	28·7	162·5	12·2	0·286	0·9775	0·9465	0·969

Distribution of ions.

We find in all our experiments (Table II) that

$$\frac{[Cl_P]}{[Cl^-_E]} > \frac{[B^+_E]}{[B_P]} \qquad \text{.....(13),}$$

or

$$\frac{[Cl_P]\,[B_P]}{[Cl^-_E]\,[B^-_P]} > 1 \qquad \text{.....(13 } a\text{)}^1.$$

Now

$$\frac{f_{Cl_P}[Cl^-_P]}{f_{Cl_E}[Cl^-_E]} = \frac{a_{Cl_P}}{a_{Cl_E}} = \frac{a_{B_E}}{a_{B_P}} = \frac{f_{B_E}[B^+_E]}{f_{B_P}[B^+_P]} \qquad \text{.....(14).}$$

It follows that either $\frac{f_{Cl_P}}{f_{Cl_E}} < \frac{f_{B_E}}{f_{B_P}}$ or the ions form undissociated compounds with protein (making $[Cl^-_P] < [Cl_P]$ or $[B^+_P] < [B_P]$).

Our experiments give us no means of estimating the actual values of $\frac{a_{Cl_P}}{a_{Cl_E}}$ or $\frac{a_{B_E}}{a_{B_P}}$; but in somewhat similar experiments Hastings, Salvesen, Sendroy, and Van Slyke found $\frac{[B_E]}{[B_P]}$ about equal to $\frac{a_{H_E}}{a_{H_P}}$.

Now

$$\frac{a_{H_E}}{a_{H_P}} = \frac{a_{B_E}}{a_{B_P}} = \frac{f_{B_E}[B^+_E]}{f_{B_P}[B^+_P]}$$

so that

$$\frac{[B^+_E]}{[B_P]} = \frac{f_{B_E}[B^+_E]}{f_{B_P}[B^+_P]} \qquad \text{.....(15),}$$

from which it appears probable that

$$f_{B_E} = f_{B_P} \qquad \text{.....(16),}$$

[1] The sum of the errors in these estimations should not exceed 2 %; the actual difference found between $[Cl_P][B_P]$ and $[Cl^-_E][B^-_E]$ always exceeds 3 %.

and that $[B_P] = [B_P^+]$, in other words that all the kation is ionised (if unionised kation were present f_{B_P} would have to be actually greater than f_{B_E}, which is improbable).

From (13), (14) and (15) we infer that $f_{Cl_P} < f_{Cl_E}$, or that $[Cl_P] > [Cl_P^-]$ (that is, that some of the Cl present in the protein solution is in an undissociated condition even on the alkaline side of the iso-electric point). Since $\frac{[Cl_P]\,[B_P]}{[Cl_E^-]\,[B_P^+]}$ decreases, if anything, with increasing p_H, it is probable that the first is the correct explanation, that is, that $f_{Cl_P} < f_{Cl_E}$.

The lowest value of $\frac{f_{Cl_P}}{f_{Cl_E}}$ so reckoned is 0·935, so that the effect of protein on the activity coefficient is not great; it appears to increase only slightly when the p_H increases from about 6·7 to about 8·0, that is when the charge on the protein is about doubled. Although the protein ion is polyvalent, the charges are probably so far apart that they have little more effect on the activity coefficients of other ions than would equivalent charges on separate ions; it is quite possible that weak electric fields round numerous polar groups at the links between individual amino-acids have more effect than do the few charges on the molecule.

The excess of diffusible ions found in the protein solution is much greater than calculated by equation (9) on the assumption that the activity coefficients of the diffusible ions were unaffected by the protein. It is not possible to reckon the osmotic pressure due to this difference simply with the aid of the calculated activity coefficients, for the osmotic coefficient involves an integration and requires a knowledge of the equation connecting activity coefficient and protein concentration; but the actual osmotic pressure due to this difference is not necessarily less than that calculated for ideal solutions.

Observed osmotic pressures.

It can be seen from Fig. 3 and Table II that the osmotic pressures found for the same serum, at the same hydrogen ion concentration and salt concentration in the external fluid, agree very well; the differences between the three sera used (two human and one horse) are what would be expected from the difference of the albumin to globulin ratios. The only result which is out of agreement is that of Exp. 4 *a*, which was obtained by the first method and the discrepancy in the case is not too great to be accounted for by the disturbances that occurred in this experiment.

Our results agree well with those of Verney [1926] and Mayrs [1926]; the osmotic pressures are uniformly higher owing to the higher temperature at which we have worked.

An increase of osmotic pressure with a rise of the p_H was detected in our experiments in which the more accurate method was used, even with low protein concentrations (Exps. 13 *c* and *d*), and with such small changes of p_H

Table II. *Osmotic pressures of serum-proteins.*

Experiment Later method	Serum	P_H ext. fluid	[Pr]	Albn. / Globn.	Osmotic pressure cm. of water 0°	37°	Remarks
9 *a*	L. H.	6·7	67·6	2·9 / 1	29·9	32·8	
9 *b*	„	6·7	40·9	2·9 / 1	15·4	17·6	
11 *a*	„	6·4	74·0	2·8 / 1	33·6	36·0	
12 *a*	„	7·5	68·6	2·8 / 1	33·2	36·8	
12 *b*	„	7·8	68·6	2·8 / 1	—	37·5	
10 *a*	„	7·8	63·6	2·9 / 1	30·1	33·65	
10 *b*	„	7·8	36·4	2·9 / 1	13·5	15·9	
8 *a*	J. M.	6·4	64·8	2·3 / 1	27·7	29·9	
8 *b*	„	6·4	21·2	2·3 / 1	—	7·4	
11 *b*	„	6·4	57·2	2·2 / 1	—	24·9	
12 *c*	„	7·5	50·3	2·2 / 1	—	22·9	
12 *d*	„	7·8	50·3	2·2 / 1	—	23·3	
13 *a*	„	6·8	62·3	2·2 / 1	27·8	29·9	
13 *b*	„	8·0	62·3	2·2 / 1	—	32·2	
13 *c*	„	6·8	38·6	2·2 / 1	15·0	15·6	
13 *d*	„	8·0	38·6	2·2 / 1	—	16·4	
Earlier method							
3 *a*	„	6·9	50·4	—	—	22·3	
5 *a*	„	6·7	63·9	2·37 / 1·00	—	30·3	
5 *b*	„	6·7	26·3	2·37 / 1·00	—	11·1	
4 *a*	„	8·3	67·8	2·40 / 1·00	—	33·3	Temperature regulation disturbed
4 *b*	„	8·3	37·1	2·40 / 1·00	—	15·8	Temperature regulation disturbed
6 *a*	„	6·4	66·9	2·31 / 1·00	—	33·3	Ext. fluid about 0·3 *M*
6 *b*	„	6·4	36·7	2·31 / 1·00	—	17·7	„ „ „
7 *a*	„	8·3	64·4	2·31 / 1·00	—	32·0	„ „ „
7 *b*	„	8·3	32·3	2·31 / 1·00	—	13·5	„ „ „
1 *a*	Horse	6·9	70·5	0·88 / 1·00	—	29·6	
1 *b*	„	6·9	42·5	0·88 / 1·00	—	15·9	
2 *a*	„	8·2	68·9	0·88 / 1·00	—	29·7	
2 *b*	„	8·2	42·6	0·88 / 1·00	—	15·7	

as that from 7·5 to 7·8 (Exps. 12 *a*, *b*, *c*, *d*). The changes of osmotic pressure found were, however, much less than those calculated for ideal solutions by equation (9). It is probable that the increase of total osmotic pressure due to the greater excess of diffusible ions in the protein solution at the higher p_H is to some extent balanced by a reduction of the activity coefficient of the protein molecules when they are more highly ionised; such a reduction of activity coefficient with increased ionisation has been found by Cohn and Prentiss [1927] in the case of haemoglobin.

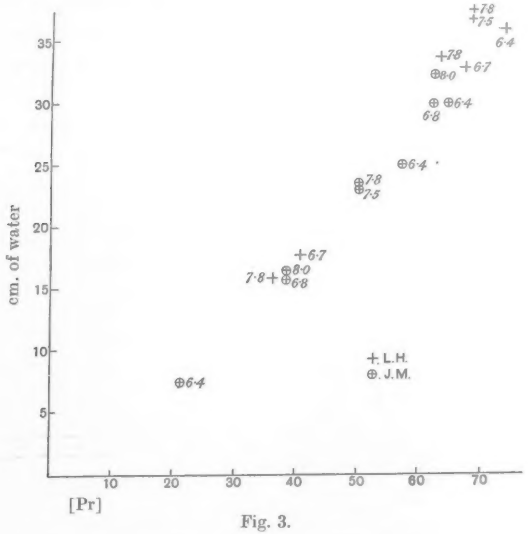

Fig. 3.

It is not therefore necessary to assume from the slight effect of change of p_H that the Donnan osmotic effect is much less than that calculated for ideal solutions and inadequate to account for a large part of the deviation of the protein concentration-osmotic pressure curve from a straight line.

With increase of the salt concentration of the external fluid the Donnan effect should be proportionately reduced, and consequently the relation between protein concentration and osmotic pressure should be more nearly linear. This we have found in Exps. 6 *a*, *b* and 7 *a*, *b*.

The changes of osmotic pressure with temperature are on the whole rather less than would be expected. The irregularity in the results is probably due to inaccuracies in the estimation at 0°, as the time allowed at this temperature was probably insufficient for the full pressure to be registered in all cases; if this is the case the lower values for $\frac{\text{O.P. at } 37°}{\text{O.P. at } 0°}$ are probably the more correct. The results at any rate suggest that there is no great change in the physical condition of the proteins in passing from 0° to 37°.

Hydration of protein.

The relation of osmotic pressure to protein concentration can be explained as shown by Verney by supposing that the protein in solution occupies a much greater volume than it occupies when dry; this could only be the case if the protein was surrounded by a large water sheath ("hydration") or enclosed by a large volume of water (as probably occurs in gelatin solution). The latter supposition can be shown (from consideration of the osmotic pressures found and the possible area of the protein molecule) to be impossible in the case of the proteins we are considering. The solubility of non-polar substances, such as nitrogen [Stoddart, 1927], in protein solutions shows that hydration of proteins, in the sense that lithium ions, for example, are hydrated, does not occur to any great extent. It may be supposed that proteins, in some unknown way, hold a sheath of water which behaves as a solvent just as does the main bulk of the water, the whole mass forming an "omnibus" of protein, water and other solutes. It must however be remembered that the chief evidence for this so-called "hydration" is based on the high viscosities of protein solutions which can be explained in other ways.

We are now further studying osmotic pressures and the effect of proteins on activity coefficients, using individual proteins.

SUMMARY.

1. The observed osmotic pressures of serum-proteins rise with increase of p_H.

2. This increase is less than that calculated for ideal solutions if the compound of protein and base is fully dissociated.

3. The activity coefficients of anions are slightly reduced by the presence of proteins in solution.

4. Both the excess of diffusible ions in the protein solution (due to the Donnan effect) and deviations from ideality will cause the observed osmotic pressures of proteins not to bear a linear relation to the protein concentration. It is not necessary to suppose that proteins in solution occupy a greater bulk than they do when dry.

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REFERENCES.

Bronsted (1921). "Fundamentals of physical chemistry." Eucken, Jette and La Mer (1925), p. 332.
Cohn and Prentiss (1927). *J. Gen. Physiol.* **8**, 619.
Hastings, Salvesen, Sendroy and Van Slyke (1927). *J. Gen. Physiol.* **8**, 701.
Mayrs (1926). *Quart. J. Med.* **19**, 273.
Scatchard (1925). *J. Amer. Chem. Soc.* **47**, 6.
Stadie and Ross (1925). *J. Biol. Chem.* **65**, 735.
Stoddart (1927). *J. Biol. Chem.* **71**, 611.
Van Slyke, Wu and MacLean (1923). *J. Biol. Chem.* **56**, 765
Verney (1926). *J. Physiol.* **61**, 319.

CLIV. EXPERIMENTS ON NUTRITION. VIII.

COMPARATIVE VITAMIN B VALUE OF FOODSTUFFS. CEREALS. I.

By ROBERT HENRY ADERS PLIMMER, JOHN LEWIS ROSEDALE (*Beit Memorial Research Fellow*), WILLIAM HENRY RAYMOND and JOHN LOWNDES.

From the Chemical Department, St Thomas's Hospital Medical School, London.

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It became evident from our previous experiments [Plimmer, Rosedale and Raymond, 1927] that all the constituents of the diet needed balancing by vitamin B to ensure satisfactory rearing of chickens. A similar balance was found in the cases of pigeons and rats. On a basal diet free from vitamin B, large quantities, such as 4 % of dried yeast, or 8 % of marmite, were found necessary to maintain pigeons for long periods.

It is essential to know not only the constituents of the diet which supply vitamin B, but also and more particularly the relative amount of it contained in each foodstuff. Cooper [1912, 1914] and Chick and Hume [1917] have given a most valuable series of comparative data, and there are a few figures by American workers. The figures of Cooper and of Chick and Hume have been expressed in terms of daily quantities on a fixed daily intake of food. From these data figures bearing a relation to the food consumption can be calculated. No quantitative information is available about the common cereals, like whole wheat, rye, maize, though data were given for wheat germ, bran, barley. There is also little quantitative information about vegetables, fruits and other foodstuffs.

These experiments have therefore been undertaken to obtain these facts about the commoner foodstuffs and thus add to our knowledge about food analysis. Some cereals and cereal products have so far been tested and experiments are in progress with other foodstuffs. The figures are purely of relative value. The exact amount of vitamin B in foodstuffs cannot be known till the substance has been isolated and identified, and even then it may not be possible for some time to devise a method for its direct estimation in foodstuffs. Meanwhile feeding experiments are required for information about nutrition.

Experimental Methods.

The vitamin B value of foodstuffs was tested by Cooper and by Chick and Hume by methods referred to as the curative and the preventive. The former gives information more rapidly and is most useful for testing the value of extracts. The results depend to a considerable extent upon the actual state of the pigeon affected by polyneuritis. Sometimes the birds rapidly reach the condition with retracted head and are easily cured; sometimes this condition is reached more slowly and the cure is more difficult. These peculiarities have also been observed by Kinnersley and Peters [1925]. The preventive method gives more exact information about a foodstuff, as its effect is observed over a considerable period, usually from 60 to 100 days. The pigeons were fed artificially with 30 to 40 g. of white rice per day and a daily quantity of the foodstuff under examination. On account of the laboriousness of the method only a few foods were tested. The results of the two methods have agreed fairly well, but there were some considerable divergences.

Our previous observations [Plimmer and Rosedale, 1923] on two pairs of pigeons on a diet of oatmeal and fishmeal have led us to adopt the preventive method. These birds were maintained for a long period and were allowed to feed *ad lib.* The food consumption amounted to 40 to 45 g. per day. They were not able to rear their young without the addition of marmite to the food. In these experiments instead of artificial feeding we have fed the birds *ad lib.* and we have taken as our standard long maintenance with rearing of young. This standard is much more severe and is possibly too high. At any rate a period of 100 days is sometimes too short. We have often observed maintenance for this time and then the birds have lost their appetite with consequent loss of weight and have died of polyneuritis. Loss of weight always accompanies loss of appetite and at present it is not yet proved that another factor than the antineuritic is concerned in their nutrition.

Foodstuffs can be placed in several groups according to the amount of vitamin B that they contain. Those with none, like white rice and white flour, those very rich, like dried yeast and wheat germ, those with intermediate quantities, which may be much or little.

Our method of testing has been to take a foodstuff, such as whole wheat, to add to it 5 % of fishmeal to supply protein and mineral salts, and see if a pair of birds could be maintained on it with reproduction. If this occurred, then a proportion of white rice (10 %) was added and reproduction once more awaited. Further dilutions of the grain or other food were then made with more white rice, until it was found that the pigeons could no longer rear their young. It was not necessary to add an antiscorbutic to the food, nor has it been found necessary to add cod-liver oil to supply fat-soluble vitamins.

This standard of testing has been found rather too severe, as in some cases there has been maintenance in perfect health without reproduction. It has been observed that if the food is satisfactory the birds eat the rice first; if

not satisfactory, the rice is left. White flour has then been substituted for white rice. This has the advantage that the food mixture can be made into a dough with water and the dough can be made into pills and dried. The birds must then either eat the whole food or leave it. The minimum quantity for maintenance on such a mixture has still to be found in several cases. The results of the experiments are given in a series of tables and additional information is supplied by a short further description.

All the birds have been kept in cages as described previously [1927] on the roof of the building, conditions which are more natural than small cages for one bird or for several. The birds were kept as quiet as possible so as to prevent neglect of their eggs and young ones.

Oatmeal.

The two pairs of adult pigeons, which had already been kept for over a year on oatmeal and fishmeal, were continued on the same diet, with or without the addition of marmite, until the end of 1923. The red pair (Nos. 1 and 2) hatched and reared three pairs of young with marmite; a fourth pair, started with marmite, died on removal of the marmite. The blue pair (Nos. 4 and 5) without marmite had five lots of eggs and failed to rear the young. On including marmite in the food, a pair of young was reared. The details about these pairs of birds are given in Table I.

Table I. *Oatmeal.*

		Diet				Weights, g.			
Date	Time in weeks	Oat-meal	Mar-mite	Cod-liver oil	Fish-meal	Cock No. 1	Hen No. 2	Number of eggs	Result
14. vi. 23		90	1·5	0	5	510	500	6 during June, July, Aug.	4 hatched and reared; 1 cracked; 1 unfertile
to								2 in Sept.	Both hatched; 1 squab died on 27th day, no cause of death found. Marmite now omitted and 2nd squab lost weight and died
2. xi. 23	20								
3. xi. 23		90	0	0	5	500	545		
to									
31. xii. 23	9					555	655		
						No. 5	No. 4		
14. vi. 23		90	0	1	5	395	390	10 during June, July, Aug., Sept.	All fertile; 7 died at hatching; 3 hatched and died
to									
17. x. 23	18								
18. x. 23		90	1·5	1	5	445	420	2 in Oct.	Both hatched and reared
to									
31. xii. 23	11					505	430		

The blue pair (Nos. 4 and 5) were continued on the oatmeal diet for the next two years with the results shown briefly in Table II. This pair was used mainly as a control for all experiments with the different cereals.

At first, 1·5 % marmite was added and a pair of young was hatched and reared for a week. The hen then unfortunately escaped and the young were deserted by the cock. With a new hen, after a single egg from which the squab died, two young were reared. Marmite was then raised to 2 %, but the eggs laid were spoilt.

Table II. *Oatmeal.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Oat-meal	Mar-mite	Fish-meal	Cock No. 5	Hen No. 4		
1. i. 24 to 18. iii. 24	11	93·5	1·5	5	480	390	4 in Jan., Feb.	1 broken; 1 unfertile; 2 hatched. Hen escaped; squabs died
						No. 6		New hen
19. iii. 24 to 27. v. 24	10	93·5	1·5	5	460	390	3 in May, June	1 hatched and died; 2 hatched and reared
28. v. 24 to 23. vii. 24	8	93	2	5	425	445	2 in July	Fertile and deserted
24. vii. 24		95	0	5	420	450	2 in Aug.	Both hatched; 1 died; 1 reared
to							2 in Feb.	1 broken; 1 died at
29. iv. 25	40				420	375		hatching
30. iv. 25 to 24. vi. 25	8				400		1 in April	Hen died; egg fertile and deserted. Cock only
						No. 4		New hen
25. vi. 25		95	0	5	400	380	12 during June, Aug., Sept., Oct., Nov.	3 unfertile; 3 hatched and reared, but poor growth and squabs unwell; 2 deserted; 2 dead in shell;
to 17. xii. 25	25				540	510		2 hatched and died

It was desired to test oatmeal alone over a long period. In 48 weeks only two lots of eggs were laid, from which only one squab was reared. After 6 months two more eggs were laid but young were not reared. A single egg was then laid and deserted. The hen was then unwell and died. The cock was also losing weight at this period. With a new hen (No. 4) one young was reared from the first two eggs, both were reared from the next eggs, and subsequently no more were reared.

Adult pigeons can thus be maintained for long periods on a diet of oatmeal and young can occasionally be reared. Young can generally be reared with an addition of marmite (2 %). The long period on oatmeal caused a slight loss of weight of the adult birds, the hen being more affected than the cock, possibly from loss of vitamin B from her body into the eggs. One hen died on the long period of oatmeal feeding.

Oatmeal would thus appear to contain just enough vitamin B for maintenance of pigeons, but not enough for rearing.

Sussex Ground Oats.

The red pair of pigeons (Nos. 1 and 2) was changed over to Sussex ground oats on 1. i. 1924. The hen laid two lots of eggs in the first period of 20 weeks, but no young were reared. In the next two periods, amounting together to 21 weeks, marmite was added. Young ones were again not reared. The cause of non-rearing seemed due rather to the fibrous nature of the food than to lack of vitamin B. The young thrived for a time and died with fibre in the gizzard. At the end of the breeding season the adult birds were kept a final 11 weeks on Sussex ground oats and both increased in weight.

Sussex ground oats are thus similar to oatmeal. Adult pigeons can be maintained, but young cannot be reared.

The details are shown briefly in Table III.

Table III. *Sussex ground oats.*

Date	Time in weeks	Diet: Sussex ground oats	Diet: Marmite	Diet: Fish-meal	Weights, g.: Cock No. 1	Weights, g.: Hen No. 2	Number of eggs	Result
1. i. 24 to 20. v. 24	20	95	0	5	495	600	4 in Mar., Apr.	3 died after hatching; 1 fertile, not hatched
21. v. 24 to 18. vii. 24	9	91	4	5	435	490	4 in May, June	1 unfertile; 3 hatched and died
19. vii. 24 to 13. x. 24	12	89	6	5	465	510	4 in July, Aug.	2 addled; 2 hatched and died in 1st week
14. x. 24 to 31. xii. 24	11	95	0	5	440 430	495 510		

Whole Oats.

The pair was changed over to whole oats for a period of one year (1925). The change had a favourable effect upon the rearing of young. Both young from the first pair of eggs were reared, one from the second lot, and one from the fourth lot. The first young ones were practically normal, but the later ones showed poor growth and were not first-class birds on removal from their parents. The adult pair maintained their healthy appearance throughout, but increased in weight too much at the end. The difference in result between Sussex ground oats and whole oats is probably due to fibre. The husk of the whole oat was usually discarded by the birds, whilst such separation was not possible from the ground oats.

Whole oats appear rather better than oatmeal. In the preparation of oatmeal, a portion of the germ with vitamin B seems to be removed.

The details are shown in Table IV.

Table IV. *Whole oats.*

Date	Time in weeks	Diet: Whole oats	Diet: Marmite	Diet: Fishmeal	Weights, g.: Cock No. 1	Weights, g.: Hen No. 2	Number of eggs	Result
1. i. 25		95	0	5	430	510	8 in Mar., May, June, July	4 hatched and reared, poor growth never quite normal; 2 addled; 2 hatched and died in 2nd week
to								
30. xii. 25	52				535	655		

BARLEY.

This pair of birds (Nos. 21 and 22) was given barleymeal for the first year (Table V). The fertility of the eggs was not good and changes in the diet had to be made after the rearing of a single squab. No squabs were produced with the diet of 70 % barleymeal. On this quantity the pair was kept for 20 weeks: no loss in weight of the birds occurred.

Table V. *Barley.*

Date	Time in weeks	Diet: Barley-meal	Diet: Rice	Diet: Fish-meal	Weights, g.: Cock No. 21	Weights, g.: Hen No. 22	Number of eggs	Result
1. i. 24		90	0	5	420	460	6 in Mar., Apr., May	2 unfertile; 2 hatched and reared; 1 fertile, deserted; 1 hatched and died
to								
1. vii. 24	25							
2. vii. 24		80	10	5	395	415	2 in June, July	1 unfertile; 1 hatched and reared
to								
12. viii. 24	7							
		Barley grains						
13. viii. 24		70	20	5	420	495	4 in Aug., Sept.	2 addled; 2 unfertile
to								
31. xii. 24	20							
1. i. 25		70	20	5	420	500	2 in Jan.	Deserted, unfertile. Cock lost
to								
10. ii. 25	7				No. 121			New cock
to					410	480		
13. iv. 25	9						2 in Feb.	2 hatched and reared
14. iv. 25		60	30	5	410	470	8 in Apr., May, July, Aug.	1 unfertile; 1 addled; 6 hatched and reared
to								
4. v. 25	3	63·5	31·5	5				
to								
8. vi. 25	5	65	30	5				
to								
5. x. 25	18							
6. x. 25		55	40	5	420	465	6 in Jan., Feb., Mar.	4 broken and deserted; 1 died at hatching; 1 died in 2nd week with symptoms of polyneuritis. Hen died, blood clot near heart
to								
26. iii. 26	24				380	480		
					No. 25/34			New hen
2. iv. 26					390	400	6 in May, June, Aug.	3 hatched and reared; 1 dead in shell; 2 fertile, and deserted
to								
20. i. 27	42				480	500		

During the second year barley grains were substituted for barleymeal. The birds had experienced some difficulty in picking up the fine meal and consequently complete consumption of the food was not really attained. With grains of barley, it was observed that all the food was picked up and eaten.

Unfortunately the cock escaped soon after this change. With a new cock, the fertility of the eggs was improved and a pair of young was reared on 70 % barley.

On reducing the barley to 60 %, then raising to 63·5 % and later to 65 %, squabs were hatched and reared.

Reduction of the barley to 55 % did not allow of rearing. One squab which was hatched died with symptoms of polyneuritis. Subsequently the hen died. With a new hen, altogether three squabs were reared—the next eggs were fertile and deserted and then no more eggs were laid. This condition of maintenance without reproduction has been observed in other cases.

55 % of barley thus seems the limiting quantity for rearing, but 65 % is better. It will be necessary to ascertain the limiting quantity for maintenance in future experiments.

Rye.

Whole rye seeds were used as food for these birds (Nos. 9 and 10). As shown in Table VI, the birds were first given rye and fishmeal. After the rearing of

Table VI. *Rye.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Rye	Rice	Fish-meal	Cock No. 10	Hen No. 9		
5. iii. 24 to 27. v. 24	12	95	0	5	375 395	415 420	2 in Apr.	Both hatched and reared
28. v. 24 to 8. vii. 24	6	80	10	5	390	420	2 in May	Both hatched and reared
9. vii. 24 to 22. iv. 25	41	70	20	5	430	435	12 in July, Aug., Sept., Oct., Jan., Mar.	10 fertile, deserted; 2 hatched and reared
23. iv. 25 to 10. vi. 25	7	60	30	5	390	415	2 in May	Both hatched and reared
11. vi. 25 to 9. ix. 25	13	63·5	31·5	5	390 450	415 425	4 in June, July	1 addled; 1 died at hatching; 2 hatched and reared
10. ix. 25 to 14. iv. 26	31	55	40	5	400	425	2 in Feb.	Both hatched and reared
15. iv. 26 to 7. vii. 26	12	40	55	5	400	405	2 in Apr.	1 fertile, deserted; 1 hatched and reared. Birds do not eat rice
8. vii. 26 to 22. ix. 26	11	45	50	5	345	370		Birds still leave rice
23. ix. 26 to 24. xi. 26	9	50	45	5	360	395		Rice eaten; birds better

a pair of squabs, rice was added in gradually increasing quantities according as young were reared. The birds were kept for the long period of 41 weeks with 70 parts of rye, mainly on account of the eggs being deserted. This was largely due to the very wet season; the nest of the birds was continually swamped with water. On reducing the rye to 60 parts, young were hatched from the first pair of eggs. Young were hatched and reared with as little as 45 % of rye. With less rye in the diet, the adult birds after a time did not eat the rice and began to look unwell. The quantity was obviously too little. No great improvement in the birds was seen on raising the rye to 45 %, but on giving 50 % rye the birds improved. It can be concluded that adult birds require 45 % rye for maintenance and that 50 to 55 % is required for rearing of young. It will be of interest in future experiments to prove definitely whether a minimum of 40 or 45 % is needed for maintenance, which will correspond with the figure found for wheat.

Wheat.

Whole wheatmeal flour, such as is used for human consumption, was given as the food for these birds (Nos. 17 and 18) (see Table VII). At first, the flour was wetted with water to bind the fine particles together, but later, on mixing with rice, the two components (the rice finely ground) and the fishmeal were made into a dough, and the dough was made into small pills which were dried on glass plates at room temperature or at 37°. Two young were reared up to a proportion of 70 % wheat. Many eggs were laid on the diet with 60 % wheat during the long period of 31 weeks, but no young were hatched. The proportion of whole wheat was then raised first to 70 % and then to 75 %. Again young were not hatched out. The hen then died. No particular symptoms could be found at *post mortem* examination and it seemed that she died of exhaustion from laying so many eggs, 30 in all.

With a new hen, keeping the whole wheat at 75 %, during the long period of 51 weeks, only two young were hatched and reared. Two other pairs of young were hatched, but they died in the second week with symptoms of polyneuritis. The other eggs were fertile, but no young were hatched from them.

It thus appeared that 75 % whole wheat was the minimum for the hatching and rearing of young, but that 80 % was distinctly better.

The quantity of whole wheat in the diet was now reduced to 60 %. The eggs which were laid were fertile, but always deserted.

In the meantime another pair of birds had been kept on 50 % whole wheatmeal (see below) and it was decided to find out what was the minimum quantity of whole wheatmeal required for the maintenance of adult birds. The quantity of whole wheatmeal was therefore reduced to 30 %. On this quantity both birds died, which showed definitely that more than 30 % was needed.

Another pair of birds (Nos. 25/2 and 25/3) was kept on 50 % whole wheat-

meal, which was mixed with white flour instead of rice, for 45 weeks. One young was hatched and reared from the first pair of eggs. The other eggs were fertile and were deserted. To test whether an increase of whole wheat-meal flour to 75 % would improve the hatching and rearing, the birds were given this diet for another 34 weeks. Rather more eggs were laid, but they were always deserted. During the time on 50 % whole wheat the birds kept a constant weight. This quantity thus seemed a satisfactory amount for long maintenance.

Table VII. *Wheat.*

Date	Time in weeks	Diet: Wheat	Rice	Fish-meal	Weights, g.: Cock No. 17	Hen No. 18	Number of eggs	Result
26. iii. 24 to 28. v. 24	10	90	0	5	440	365	4 in Apr., May	2 hatched and reared; 2 deserted
29. v. 24 to 23. vii. 24	8	80	10	5	430	370	2 in June	Both hatched and reared
24. vii. 24 to 3. ix. 24	6	70	20	5	425	365	4 in July, Aug.	2 hatched and reared; 2 deserted
4. ix. 24 to 7. iv. 25	31	60	30	5	440	365	14 in Sept., Oct., Nov., Jan., Feb., Mar., Apr.	4 unfertile; 10 addled and deserted
8. iv. 25 to 6. v. 25	4	70	20	5	450	385	2 in May	1 unfertile; 1 fertile, deserted
7. v. 25 to 17. vi. 25	6	75	20	5	445	380	4 in May, June	1 very small egg; 2 unfertile; 1 addled. Hen died; no particular symptoms
					No. 118			New hen
1. vii. 25 to 26. vi. 26	51	75	20	5	400	335	12 in July, Oct., Feb., Mar., May	1 addled; 2 hatched and reared; 2 hatched and died in 2nd week with enlarged hearts; 4 deserted; 2 dead in shell; 1 hatched, died in 2nd week with polyneuritis
					430	365		
27. vi. 26 to 29. ix. 26	14	60	35	5	450	415	7 in June, July, Aug., Sept.	5 deserted, fertile; 2 dead at hatch
30. ix. 26 to 7. iii. 27	23	30	65	5	350		2 in Oct.	Deserted, fertile. Hen died on 31. xii. 26 after 13 weeks. Cock died of polyneuritis
					No. 25/2	No. 25/3		
18. xi. 25 to 29. ix. 26	45	50	45	5	440	445	8 in Feb., Apr., June, July	1 dead in shell; 1 hatched and reared; 1 hatched and died; 5 fertile and deserted
					415	465		
30. ix. 26 to 25. v. 27	34	75	20	5	435	460	9 in Oct., Feb., Mar., Apr., May	8 fertile and deserted 1 unfertile
					No. 93	No. 26/27		
10. iv. 27 to 17. viii. 27	23	40	55	5	455	380	6 in Apr., May, June	1 hatched and reared, but very poor growth; 2 unfertile; 3 fertile and deserted
					385	295		

It remained to ascertain if 40 % whole wheatmeal would be enough for maintenance. Another pair of birds (Nos. 93 and 26/27) was started on this quantity. They managed to rear one young one from the first lot of eggs, but this young one showed very poor growth and was very small. Two other lots of eggs were laid which were deserted and then no more. They have been kept for over 20 weeks without signs of illness, but they have shown a slight loss in weight.

It may be concluded that 40 % of whole wheat is the minimum for maintenance of adult pigeons, 50 % is a more favourable quantity and 75 to 80 % whole wheat is required for reproduction and rearing of young.

Wheat Germ.

The high vitamin B value of wheat germ was determined by Chick and Hume [1917]. They found that pigeons on a daily diet of 30 to 40 g. of white rice were protected over a period of 63 days by a dose of 3 g. of wheat germ given every second day. This amount corresponds to about 4·5 % of the diet. According to our experiments the smallest quantity which must be present in the diet is 6 %. Table VIII shows our results. The first period was for

Table VIII. *Wheat germ.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		White rice	Wheat germ	Fish-meal	Cock No. 24/13	Hen No. 24/10		
23. vii. 24 to 11. xi. 24	16	90	10	5	360	330		
12. xi. 24 to 17. ii. 25	14	90	6	5	445	395		Birds found to be two hens
		White flour			No. 31	No. 24/10		
18. ii. 25 to 30. vi. 25	19	89	6	5	460	440	8 in Feb., Mar., Apr., May, June	1 unfertile; 2 addled; 2 dead at hatching; 3 fertile and deserted
1. vii. 25 to 20. xi. 25	20	87	8	5	425	385	6 in July, Aug.	All deserted. 24. viii. 25, hen ill, dosed with 4 doses of 2 g. wheat germ; better. Hen died with polyneuritis; enlarged heart
						No. 25/31		New hen
25. xi. 25 to 1. vi. 26	27	85	10	5	460	460	4 in Mar., Apr.	Fertile and deserted
2. vi. 26 to 27. vii. 26	8	93	2	5	430	395	1 in June	Fertile, deserted. Hen died 7. vii. 26, in 5 weeks. Cock died
					No. 26/2	No. 26/1		
8. vii. 26 to 22. ix. 26	11	91	4	5	345	395	2 in July	Deserted. Both birds died of polyneuritis

16 weeks with a proportion of 10 % of wheat germ and was followed by a period of 14 weeks with 6 %. The supposed cock of this pair of birds was then discovered to be a hen. On exchanging one of the hens for a cock the pair was kept for 19 weeks on 6 % of wheat germ. At this time white flour was used instead of white rice, and the diet given in small pills.

The hen laid eggs during this period, but they were always deserted. The quantity of wheat germ was then raised to 8 % to see if any improvement in hatching would take place. The hen showed signs of polyneuritis after 8 weeks, but was cured with four doses of 2 g. of wheat germ. At 20 weeks the hen again suffered from polyneuritis and died.

Another hen was placed with the cock and the amount of wheat germ was raised to 10 %. The hatching was no better and after 27 weeks the attempt to produce rearing was discontinued.

It was decided to find out the smallest quantity of wheat germ which would suffice to maintain a pair of pigeons. The quantity was therefore reduced to 2 %. Both birds died in 8 weeks.

Table IX. *Heated wheat germ.*

		Diet			Weights, g.			
Date	Time in weeks	White rice	Heated wheat germ	Fish-meal	Cock No. 24/12	Hen No. 24/14	Number of eggs	Result
23. vii. 24		90	10	5	315	370		Cock lost
to					No. 9131			New bird
11. xi. 24	16				430	440		
12. xi. 24		90	6	5	365	430		New bird found to be hen
to								
11. ii. 25	13				450	450		
to					No. 25			Another cock
					380	410		Hen died from unknown cause
4. iii. 25	3				390	395		
		White flour				No. 24/23		New hen
11. iii. 25		89	6	5	375	370	6 in Apr., May, June	4 fertile and deserted; 1 addled; 1 died at hatching
to								
30. vi. 25	16							
1. vii. 25		87	8	5	405	395	10 in July, Aug., Sept., Oct.	2 deserted; 1 unfertile; 5 hatched and died; 2 addled
to								
18. xi. 25	20				430	430		
19. xi. 25		85	10	5			14 in Nov., Dec., Jan., Feb., Mar., Apr.	5 unfertile; 3 fertile and deserted; 5 broken; 1 addled
to								
12. v. 26	25				390	400		
13. v. 26		83	12	5			2 in May, June	1 egg only at each time of laying; both broken
to								
28. vii. 26	10							Cock escaped
					No. 26/6	No. 26/3		
27. vii. 26		91	4	5	405	385		Cock died in 6th week; hen died in 8th week
to								
22. ix. 26	8							

Another pair was started on 4 % wheat germ. These died in 11 weeks. 2 % and 4 % wheat germ were therefore insufficient to balance the white flour. On 6 % two birds were kept for 14 weeks and another pair for 19 weeks. One of the two had been for both these periods on this quantity. 6 % must therefore be taken as the minimum quantity for long maintenance.

Heated Wheat Germ.

At the same time as the experiments with wheat germ were being made, similar ones with heated wheat germ were carried out. The wheat germ is heated to about 120° to prevent its decomposition. Table IX shows the results. A pair of birds was kept for 16 weeks with 6 % heated wheat germ. There was a slight improvement in hatching with 8 and 10 % wheat germ.

A pair of birds on 4 % of heated wheat germ died in 8 weeks. For maintenance therefore 6 % of wheat germ is required. The germ does not lose vitamin B by this treatment[1].

Bran and Middlings.

The high content of vitamin B in bran was found by Chick and Hume [1917]. It appeared to be about one-fifth of that of wheat germ.

Table X. *Bran.*

Date	Time in weeks	Diet				Weights, g.		Number of eggs	Result
		Bran	White flour	Fish-meal	Mar-mite	Cock No. 24/4	Hen No. 24/6		
9. vii. 24 to 2. ix. 24	8	45	45	5	0	365	365		
3. ix. 24 to 14. iv. 25	32	30	60	5	0	395	455	6 in Jan., Feb., Mar.	4 died at hatching; 1 addled; 1 fertile, deserted
15. iv. 25 to 22. ix. 25	23	47·5	47·5	5	0	430	425	10 in Apr., May, June, July, Aug., Sept.	3 died at hatching; 2 unfertile; 2 addled; 3 hatched and died
23. ix. 25 to 2. ii. 26	19	33	60	5	2	420	450	4 in Oct., Jan.	2 hatched, 1 died 1 reared; 2 fertile and deserted
3. ii. 26 to 18. v. 26	15	33·3	57·7	5	4	430	455	4 in Feb., Apr.	2 unfertile, deserted; 2 addled, deserted
19. v. 26 to 22. vi. 26	5	33·3	55·7	5	6	385	410	2 in May	Deserted
					Dried yeast				
23. vi. 26 to 29. ix. 26	14	33·3	55·7	5	6	380 415	400 425	5 in July, Sept.	1 egg only and deserted; 4 fertile and deserted

[1] The wheat germ and heated wheat germ were kindly supplied by Mr W. T. Ryle. Messrs Hovis, Ltd., have also kindly given us wheat germ for these experiments.

Table XI. *Middlings.*

Date	Time in weeks	Diet: Middlings	Diet: White rice	Diet: Fish-meal	Diet: Marmite	Weights, g.: Cock No. 24/3	Weights, g.: Hen No. 24/5	Number of eggs	Result
9. vii. 24 to 2. ix. 24	8	70	20	5	0	370	335		
3. ix. 24 to 9. xii. 24	14	60	30	5	0	365	355		
10. xii. 24 to 31. iii. 25	16	30	60	5	0	415	385	4 in Feb., Mar.	1 unfertile; 1 addled; 2 dead at hatching
			White flour						
1. iv. 25 to 16. vi. 25	11	30	60	5	0·6	435	380	5 in Apr., May, June	1 deserted; 2 addled; 1 unfertile; 1 died at hatching
17. vi. 25 to 13. i. 26	30	33·3	60	5	1·7	405	375	10 in June, July, Aug., Sept., Nov.	4 deserted; 2 hatched and reared; 4 hatched and died
14. i. 26 to 5. v. 26	16	33·3	58·7	5	3·0	495	440	8 in Jan., Feb., Mar., Apr.	2 dead at hatch; 6 broken, deserted
6. v. 26 to 23. vi. 26	7	33·3	55·7	5	6·0	435	375	4 in May, June	Broken and deserted
					Dried yeast				
24. vi. 26 to 29. ix. 26	14				6·0	395 430	335 360	2 in July	Broken. Hen ill 30. viii. 26

In order to obtain further comparative figures for these foodstuffs pairs of pigeons were started on 45 % bran and on 70 % middlings. As shown in Tables X and XI, the pair was kept on 30 % bran for 32 weeks. As they were not able to rear young the amount of bran was raised to 47·5 %. Some eggs were hatched, but the young died. On account of the large amount of fibre in bran, extra vitamin B was supplied by incorporating a proportion of marmite; one young squab was then reared. With a further increase of marmite, no more young were reared and there was no improvement on changing from marmite to dried yeast. The attempt to rear was then abandoned.

Similar results were obtained with middlings. There was better hatching and rearing on adding marmite to the diet. With still more marmite, there was no better rearing. It would appear that some other factor than vitamin B was now concerned in reproduction. Both with bran and middlings the weights of the parent birds remained practically constant.

As far as maintenance of adult pigeons is concerned, the pair was kept on 30 % bran for 32 weeks, and the pair on 30 % middlings for 16 weeks. In future experiments it is proposed to ascertain the smallest quantities of bran and middlings upon which adult pigeons can be maintained for 20 to 26 weeks.

Maize.

Kibbled maize (see Table XII) was used as food for these pigeons (Nos. 13 and 14). At first, partly owing to the wet weather, the eggs were deserted. An addition of marmite was made to the food and ultimately a squab was reared.

Table XII. *Maize.*

		Diet			Weights, g.			
Date	Time in weeks	Kibbled maize	Marmite	Fishmeal	Cock No. 14	Hen No. 13	Number of eggs	Result
19. iv. 24 to 27. vi. 24	10	90	0	5	445	385	6 in Apr., May, June	4 fertile and deserted; 2 addled
28. vi. 24 to 8. viii. 24	9	90	2	5	455	400	2 in July	Addled
9. viii. 24 to 24. x. 24	11	90	3	5	465	420	2 in Sept.	1 addled; 1 hatched and reared
25. x. 24 to 29. v. 25	31	90	0	5	500	440	14 in Oct., Nov., Dec., Jan., Mar., Apr.	4 spoilt by wet weather; 4 fertile and deserted; 1 addled; 5 hatched and reared
			White rice					
30. v. 25 to 4. ix. 25	14	85	5	5	435	395	8 in June, July, Aug.	4 hatched and reared; 2 deserted; 2 hatched and died
5. ix. 25 to 30. ix. 26	56	75	20	5	470	415	23 in Oct., Nov., Jan., Feb., Mar., Apr., May, June, July, Aug.	6 hatched and reared; 6 unfertile; 4 addled; 7 fertile and deserted
1. x. 26 to 9. iii. 27	23	50	45	5	450	410	16 in Oct., Nov., Dec., Jan., Feb.	6 fertile and deserted; 10 broken
10. iii. 27 to 1. vi. 27	9	95	0	5	495 435	425 395	7 in Mar., Apr., May	All broken
					No. 26/4	No. 26/5		
29. vii. 26 to 16. iii. 27	33	60	35	5	410 455	355 410	6 in Jan., Feb., Mar.	3 fertile and deserted; 1 hatched and died; 2 dead at hatch
			White flour					
17. iii. 27 to 20. vii. 27	18	60	35	5	435	390	7 in Apr., May, June, July	5 fertile and deserted; 1 fertile and dead at hatch; 1 hatched and died

On returning to the simple maize and fishmeal diet, several young were hatched and reared. The maize thus appeared to contain enough vitamin B for rearing. After adding white rice to the diet, young were hatched and

reared. The pair was kept on 75 % kibbled maize for the long period of 56 weeks during which time 6 squabs were reared. This proportion of maize thus seemed the limit for rearing. No young were reared on 50 % maize, though the parents were kept on it for 23 weeks. On returning to kibbled maize only, rearing was not improved. After the long period of over two years the experiment with these birds was not continued. Lack of the power of rearing was probably due to exhaustion of some other factor which is required.

Another pair of pigeons was in the meantime tried on 60 % kibbled maize. Though many eggs were laid, and some hatched out, there was no rearing of the young.

75 % maize must be regarded as the minimum for rearing. So far 50 % has been found enough for maintenance, but 60 % was distinctly better. Future experiments are designed to find out if pigeons can be maintained on less.

Maize Germ-meal and Maize Gluten.

These two cereal products are often used in feeding poultry and other animals.

The high vitamin B value of maize germ, picked by hand from the grain, has been shown by Chick and Hume [1917]. It was consequently expected that maize germmeal would be rich in vitamin B.

A pair of pigeons was started on 90 parts rice, 10 parts maize germ-meal and 5 parts fishmeal. They lost weight at once and looked ill. On raising the maize germ-meal to 20 parts, there was no improvement and the birds died in altogether 11 weeks.

On trying a pair of pigeons on 90 parts maize germ-meal and 5 parts of fishmeal, they became ill and died in 6 weeks.

Another pair was tried with 90 parts maize germ-meal, 90 parts white flour and 5 parts fishmeal. They also became ill, but recovered on adding 5 % of marmite to the mixture. On reducing the marmite to 3 parts, the quantity to balance the white flour, they became ill and died.

Commercial maize germ-meal thus appears to contain no, or very little, vitamin B.

Maize gluten was not expected to contain vitamin B. The following experiments showed the presence of a small amount. A pair of birds was given 45 parts maize gluten, 45 parts white flour, 5 parts fishmeal and 5 parts marmite. They showed growth for 6 weeks, but on reducing the marmite to 3 parts they lost weight and died.

A pair of birds on 90 parts maize gluten and 5 parts fishmeal died in 4 and 14 weeks.

Maize gluten may thus contain small amounts of vitamin B.

Buckwheat.

Buckwheat, often used in America for human consumption and in France for feeding poultry, has been tested on the pair of pigeons Nos. 23 and 24

(Table XIII). After 18 weeks on buckwheat alone, a pair of squabs was reared. One young one was reared on 80 parts of buckwheat. The hen subsequently laid no more eggs. The pair was maintained without loss of weight for 31 weeks. The cock then died. With a new cock there was no improvement, even after returning to buckwheat alone as the diet. Finally a small proportion of cod-liver oil was added; eggs were still not laid by the hen. This grain seems to differ from the other grains by not permitting the laying of the eggs by the hen.

Further experiments are needed to ascertain the peculiarities of buckwheat.

Table XIII. *Buckwheat.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Buck-wheat	White rice	Fish-meal	Cock No. 23	Hen No. 24		
20. ii. 24 to 25. vi. 24	18	90	0	5	420	285	7 in Mar., Apr., May	1 little egg; 2 shell-less; 1 hatched and died; 3 hatched and reared
26. vi. 24 to 5. viii. 24	6	80	10	5	415	300	2 in June	1 unfertile; 1 hatched and reared
6. viii. 24 to 11. iii. 25	31	70	20	5	450 395	330 340	No eggs	Cock died; tumour
12. iii. 25 to 16. ix. 25	35	95	0	5	365 400	340 335	No eggs	New cock
17. ix. 25 to 6. xii. 25	12	94·5	0	5	435	345	No eggs	With 0·5 % cod-liver oil

Millet and Dari.

These two grains much used as human food by the natives of South Africa with good results showed a high vitamin B value on testing on pigeons. Tables XIV and XV show the behaviour of the pigeons. On gradually reducing the proportion of millet in the diet, very good hatching and rearing took place with 60 parts of millet. With 50 parts of millet the rearing was less good, but was slightly improved with 55 parts. The minimum for rearing can be said to be 55 %. Smaller quantities to determine the figure for maintenance have not yet been tried. The birds have been quite well on 50 % for 18 weeks.

The pair of birds on dari were quite prolific, but the fertility of their eggs was not good. A pair of squabs was reared on 50 %. The quantity for rearing is more certain at 60 %.

The maintenance quantity has not been determined, but experiments for this purpose will be undertaken shortly. The pair of birds lived quite well on 50 % dari.

Table XIV. *Millet.*

Date	Time in weeks	Diet: Millet	Diet: White rice	Diet: Fish-meal	Weights, g.: Cock No. 15	Weights, g.: Hen No. 16	Number of eggs	Result
1. v. 24 to 25. vi. 24	8	90	0	5	350	380	2 in May	Both hatched and reared
26. vi. 24 to 26. viii. 24	9	80	10	5	375	365	4 in June, July	2 addled; 2 hatched and reared
27. viii. 24 to 22. x. 24	8	70	20	5	415	395	2 in Aug., Sept.	1 dead in shell; 1 hatched and reared
23. x. 24 to 31. xii. 24	10	60	30	5	430	400	2 in Nov.	Both hatched and reared
1. i. 25 to 5. v. 25	18	50	40	5	480 420	425 415	6 in Jan., Mar., Apr.	1 dead in shell; 1 unfertile; 2 hatched and reared; 2 hatched and died
6. v. 25 to 9. vi. 25	5	53	42	5			2 in May	1 dead in shell; 1 hatched and died
10. vi. 25 to 12. x. 25	18	55	40	5	395	380	6 in June, July, Sept.	4 dead in shell; 1 hatched and reared; 1 unfertile
13. x. 25 to 23. xi. 25	6	60	35	5	435	430	2 in Oct.	Both hatched and reared
24. xi. 25 to 25. i. 26	9	55	40	5	440 495	435 430	2 in Dec.	Both hatched and reared

Baker's Yeast.

Our experience with dried yeast and marmite led to testing the value of baker's yeast. Analysis of a sample showed 2·23 % of N (= 13·9 % of protein), 1·9 % of ash and 71·3 % of water, which would indicate a value of about one-third of dried yeast.

An experiment was started with 10 % of baker's yeast. The birds at first increased slightly in weight, then remained constant for about 20 weeks. During this time the hen laid three lots of eggs. They were fertile, but were deserted. Subsequently, both birds lost their appetite and their weight and died at the end of the 26th week with the typical retracted head symptoms of polyneuritis. Table XVI gives the further information.

Another experiment was made with 4 % of baker's yeast, the amount corresponding with the necessary amount of dried yeast. The birds did not like the food and scattered it. They lost weight at once and both showed polyneuritis in the fifth week. The cock was cured by a dose of 2 g. of wheat germ, but the hen was allowed to die.

Table XV. *Dari.*

Date	Time in weeks	Diet: Dari	Diet: White rice	Diet: Fish-meal	Weights, g.: Cock No. 7	Weights, g.: Hen No. 8	Number of eggs	Result
20. ii. 24 to 23. vi. 24	18	90	0	5	400	420	7 in Mar., Apr., May, June	2 unfertile; 2 fertile and deserted; 2 deserted; 1 hatched and reared
24. vi. 24 to 8. viii. 24	8	80	10	5	475	380	2 in July	Both hatched and reared
19. viii. 24 to 6. x. 24	7	70	20	5	490	395	2 in Aug.	1 died at hatching; 1 hatched and reared
7. x. 24 to 17. xi. 24	6	60	30	5	490	435	2 in Oct.	1 died at hatching; 1 hatched and reared
18. xi. 24 to 6. iv. 25	20	50	40	5	570	440	6 in Dec., Jan., Feb.	3 unfertile; 1 fertile and deserted; 2 hatched and reared
7. iv. 25 to 8. vi. 25	9	45	45	5	520	415	4 in Mar., May	3 hatched and reared; 1 unfertile
9. vi. 25 to 30. vi. 25	3	50	45	5	450	400	2 in June	Both died at hatching
1. vii. 25 to 13. x. 25	15	55	40	5	440	380	6 in July, Aug., Sept.	1 hatched and reared; 1 unfertile; 2 addled; 2 dead at hatching
14. x. 25 to 24. xi. 25	6	60	35	5	500	440	2 in Oct.	Both hatched and reared
25. xi. 25 to 5. i. 26	6	55	40	5	530 590	410 450	2 in Dec.	Both unfertile

Table XVI. *Baker's yeast.*

Date	Time in weeks	Diet: White flour	Diet: Baker's yeast	Diet: Fish-meal	Weights, g.: Cock No. 53	Weights, g.: Hen No. 48	Number of eggs	Result
16. ii. 27		85	10	5	415	320	6 in Apr., May, June	All fertile and deserted.
					440	365		
to					400	320		
					410	335		
17. viii. 27	26				340	220		Both birds died of polyneuritis

Discussion and Summary.

At the time that these experiments were started, it was generally understood that the term vitamin B applied to the antineuritic substance preventing beri-beri. Yeast and other foods have sometimes been described as possibly containing two unknown food factors, the one antineuritic and the other maintaining body weight and necessary for growth. The work of Goldberger and his colleagues, and, more recently, that of Chick and Roscoe [1927] again tend to differentiate two factors in yeast and wheat embryo. The one factor

is definitely antineuritic and can be destroyed by heating at 120° under pressure; the other is thermostable, preventive of pellagra and maintains body weight and is called by Goldberger the PP factor. Chick and Roscoe wish to call this PP factor vitamin B. The nomenclature of the various unknowns in nutrition is already sufficiently complicated, *e.g.* two D vitamins have been described; the one water-soluble in yeast (Funk) and the other fat-soluble, now more definitely called vitamin D. Funk's vitamin D may be the same as the PP factor. It would have been simpler to leave the term vitamin B for the antineuritic substance with which it has so long been associated and give the term PP factor to the unknown substance in yeast and other foods which prevents pellagra and maintains body weight.

The term antineuritic limits the symptoms to those of paralysis, but a deficiency of this factor is responsible for a whole sequence of other symptoms relating to the alimentary tract, the endocrine organs and the heart, as shown by McCarrison, and observed by us on feeding with too little yeast [1927]. The animal grows, showing the presence of the second factor, but dies with alimentary, heart, or paralytic symptoms according to the degree of shortage of the vitamin.

The existence of two forms of beri-beri, the wet and the dry, has long been known, and it has often been suggested that they were due to two factors. Vedder was never able to produce the dropsical condition in birds, even with extra salts in the diet. Our observations again point to the different forms as being due to the degree of shortage of vitamin B. We have often seen wet tissues in our birds (more often in chickens) when the degree of shortage is slight and exists over a long period. A great shortage causes an early death and the tissues are dry. Before death the bird has generally stoppage of the cloaca. If excretion should occur, the birds get better for a time. If a bird is cured with a dose of yeast or wheat germ, there is first of all excretion and then recovery.

With reference to the unknown factor concerned in preventing pellagra, it is not definitely proved that it is not a constituent of proteins, as Goldberger originally believed. The experimental work of Chick and Roscoe was done with caseinogen, a protein which cannot be looked upon as a complete one. Caseinogen is deficient in cystine and may also lack other protein units. These deficiencies are probably compensated for by lactalbumin in milk, which has been shown to be so efficacious in preventing and curing pellagra and also promotes growth. Yeast protein may contain the same unit and similarly wheat embryo, especially the less pure samples which are better for pellagra prevention and for maintaining body weight. It would be of great interest to know if the same results are obtained with lactalbumin, or other complete protein. The protein in our experiments has been fishmeal.

In any case we hope that Chick and Roscoe will consider our plea to continue the association of the term vitamin B with the antineuritic factor and adopt the term PP factor for the unknown factor in yeast concerned in

pellagra prevention and in maintaining body weight. McCollum and Davis associated the antineuritic substance with vitamin B more particularly from the fact that impure lactose contained vitamin B and would cure polyneuritis in pigeons, rather than from the similar distribution of the substances.

Our results are considered entirely in respect of the antineuritic substance. We have observed previously [1927] that young birds and rats need more of it than adult animals. These data with cereals support those observations. Adult pigeons can be maintained on a smaller quantity of cereal than that upon which young can be reared. The same argument can be applied to the PP factor, that young animals want more than adults, but against this contention is the fact that the young bird grows quite rapidly and then dies showing symptoms of polyneuritis, so that it appears to have enough PP factor. Our observations have also always been to the effect that the bird, whether young or adult, first loses its appetite with corresponding loss of weight and then dies of polyneuritis. The quantity of antineuritic factor is the determining consideration.

Our data may be summarised very simply as follows:

	Percentage quantity of foodstuff required for	
	Rearing	Maintenance
Oatmeal	More than 95	95
Sussex ground oats ...	More than 95	95
Whole oats	95	95
Barley (whole) ...	65	55
Rye (whole)	50–55	45
Whole wheat flour ...	75–80	40–50
Wheat germ	More than 10	6
Bran	—	33
Middlings	—	33
Maize (kibbled) ...	75	50–60
Maize germ-meal ...	Contain very little vitamin B	
Maize gluten		
Buckwheat	80	70
Millet	55	50
Dari	60	50
Dried yeast	—	4
Baker's yeast	—	10–12
Marmite	—	8–10

As pointed out previously, some of the quantities for maintenance may be less than shown in this table. It is curious to see that the common cereal grains, wheat, rye, barley, have about the same value, that millet and dari have a rather greater value, maize a little less, and that oatmeal is least good in respect of vitamin B.

In general, our figures for maintenace are higher than those calculated from the data of Chick and Hume and of Cooper. The explanation is that our experiments were for a longer duration than 100 days. We have frequently seen polyneuritis on an amount corresponding to that of Chick and Hume after 15 weeks and occasionally after 20 or even 25 weeks. The minimum quantity adopted by us is that which keeps the birds in health for 26 or more weeks.

The relative value of the different cereals is shown better by adopting one of the above foods as a standard. It is most convenient to adopt the food richest in vitamin B, namely dried yeast, and give it the figure of 100. Then, by putting any of the above amounts for maintenance equal to 4 (dried yeast), the relative amount is given by calculating for 100 of the cereal.

Thus: 40 whole wheat = 4 dried yeast, therefore 100 whole wheat = 10; 50 whole wheat = 4 dried yeast, therefore 100 whole wheat = 8; 6 wheat germ = 4 dried yeast, therefore 100 wheat germ = 66.

The series of relative values is then as follows:

Relative vitamin B values of foods.

Dried yeast	100	Rye	9	Bran	12–13
Baker's yeast	33–40	Barley	7–8	Middlings	12–13
Marmite	40–50	Oats	4–5	Maize	7–8
Wheat germ	66	Oatmeal	4	Millet	8
Whole wheat	8–10	Buckwheat	5–6	Dari	8

It is further not difficult to calculate from these relative figures the quantity of food that must be contained in a diet. By dividing 400 by the above figures one obtains the percentage amount that should be included in a diet otherwise devoid of vitamin B. Another way of determining the amount of cereal, or a mixture of cereals, that should be present in a diet is by including the quantity, or quantities, that supply enough to give a total of 4. Thus, 20 % of millet will give 1·6, 10 % of dari will give 0·8, 40 % of whole wheat will give 3·2. Total = 5·6. The remainder of the diet can be white rice, or white flour, or fishmeal or any foodstuff without vitamin B.

These figures are for pigeons. For chickens, the amount must be calculated on a basis of 6, instead of 4. For rats, the vitamin B requirement is less, about half that of the pigeon, so that its amount will be calculated on a total of 2. The chief question is the requirement of man. He can be considered like a rat with the figure of 2, but it would be safer to consider that his requirement approaches that of a pigeon. The only figure that we can deduce for man is based on the experience in the Japanese Navy. Beri-beri was prevented by a ration of barley amounting to approximately one-third of the dry weight of the total food. This quantity, if it be assumed to be a minimum, gives a figure of not more than 3 for man's requirement, between that of a rat and a pigeon.

We acknowledge with thanks a grant from the Government Grant Committee of the Royal Society which has in part defrayed the cost of these experiments.

REFERENCES.

Chick and Hume (1917). *Proc. Roy. Soc. Lond.* B, **90**, 44, 60.
Chick and Roscoe (1927). *Biochem. J.* **21**, 698.
Cooper (1912, 1914). *J. Hyg.* **12**, 436; **14**, 12.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
Plimmer and Rosedale (1923). *Biochem. J.* **17**, 787.
Plimmer, Rosedale and Raymond (1927). *Biochem. J.* **21**, 913

CLV. THE ESTIMATION OF CYANOGENETIC GLUCOSIDES.

By LAURENCE ROBERT BISHOP.

From the Botany School, Cambridge.

(*Received August 30th, 1927.*)

As a preliminary to a study of the behaviour of the cyanogenetic glucoside (prulaurasin) in the leaves of cherry laurel (*Prunus laurocerasus*) an attempt was made, at the suggestion of Dr F. F. Blackman, F.R.S., to find a method for the estimation of the glucoside in a single leaf or half leaf. After a trial of various methods the most satisfactory was found to be by decomposition of most of the glucoside by heating the leaf in water and by decomposition of the remainder by added emulsin. During both processes a current of air was passed through the generating vessel into potash. The HCN liberated during the decomposition and absorbed in the potash was titrated in the alkaline solution with silver nitrate (Liebig's method).

Apparatus.

This was composed of six units each connected by equal lengths of fine bore capillary tubing (to equalise the bubbling rates) to a bottle exhausted by a good filter pump. The six generating vessels, 500 cc. filter flasks, were placed in a water-bath which could be heated to boiling and cooled by changing the water.

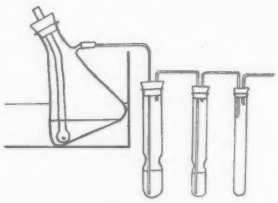


Fig. 1.

Each generating flask (Fig. 1) had an inlet tube through the stopper with fine openings below the surface of about 100 cc. of water in the bottom of the tilted flask. The side tube of the flask was connected to two test-tubes, in series, with their inlet tubes reaching to the bottom and perforated there with fine holes. The first test-tube was 3 cm. in diameter and contained 15 cc. of 5 % potash, and the second 1·5 cm. in diameter and contained 5 cc. of potash. Each of these test-tubes had a constriction just above the level of the liquid

contained in it, which served as a means of checking frothing and promoting circulation. The outlet tube of the second test-tube was connected to a third with short inlet and outlet tubes. This served to catch any potash which might have frothed over. The last test-tube was connected to the equalising resistance tubing. The contact with rubber tubing was made as small as possible.

Method.

The air current is turned on so as to produce violent bubbling and the first of the weighed leaves is cut up into small narrow pieces as quickly as possible, on a sheet of glazed cardboard, by means of a sharp scalpel. The pieces are then rapidly pushed into the first flask. The other five leaves are treated in a similar manner and put into their respective flasks and the temperature of the bath is raised to 100° during a quarter to half an hour and kept at this temperature for several minutes. The heating is then stopped and the temperature of the bath lowered by running in cold water and running out the hot. About 0·05 g. of emulsin is then added to each flask together with a few drops of amyl alcohol to check frothing.

The time during which the air current should be continued varies with the air rate and other peculiarities of the apparatus and is best determined from a curve of the rate of exhaustion of HCN from amounts of amygdalin similar to those of the glucoside to be estimated. With the apparatus described 12 hours appeared sufficient, so that in practice 14–18 hours' run was given.

Titration of the HCN. The contents of the second and third test-tubes are washed into the first. The resulting volume of liquid (about 25 cc.) is approximately halved by the constriction in the tube. Advantage is taken of this to obtain rapid titration. The tube is placed in a box with three sides and the floor blackened inside and the fourth side open and facing a source of bright diffused light. A standard amount (about five drops) of 5 % potassium iodide solution is added and $M/100$ silver nitrate is run in from a 10 cc. microburette. The silver nitrate is mixed, by means of a stirring rod flattened at the end, with the upper half of the solution only, and this can be titrated quickly to a slight turbidity. A further amount of silver nitrate almost equal to the first is then run in and the whole mixed. By repeating this process of titrating the upper half and then mixing the whole solution, the end-point (a very slight turbidity) can be reached rapidly without fear of over-titrating. The blackened box ensures that the first trace of turbidity will be recognised by means of the Tyndall effect. The potassium iodide must be added since the amounts of HCN are here too small for the precipitation of AgCN to act as an indicator. In the presence of KI the turbidity is due to the precipitation of AgI immediately there is excess of silver nitrate over that required to form the double compound AgCN.KCN.

1 cc. of $M/100$ silver nitrate = 0·0005404 g. of HCN or 0·00590 g. of prulaurasin.

Discussion of Various Methods.

Estimation of the glucoside by means of the benzaldehyde liberated was rejected owing to the liability of this substance to oxidation [Armstrong and Horton, 1908].

Estimation of the glucoside sugars by the amount of change in optical rotation or copper-reducing power after the addition of enzyme involves the extraction of the glucoside before estimation. Complete extraction is difficult and it will be shown later that dropping the leaves into boiling absolute alcohol, to kill them before the estimation, involves a loss of about 6 % of the glucoside, judged by the liberation of HCN which takes place at this stage.

Distillation method.

Evidence has been produced by several workers [Alsberg and Black, 1916; Willaman, 1917] that the distillation of plant tissue with acid results in incomplete recovery of the HCN from the cyanogenetic glucosides.

This was confirmed by comparison with the aeration method, using cherry laurel leaves. Direct distillation with acid gave a recovery of 27 % and distillation after maceration 14 %. Distillation without acid gave 49 % and subsequent distillation with acid a further 3 %. These recoveries are calculated by taking as 100 % the HCN content, determined by the aeration method, of the corresponding halves of the leaves.

It can be seen from this that the distillation method is not a reliable quantitative method. Distillation without acid gives higher yields than with it.

The distillation of small amounts of amygdalin with acid gave similar incomplete yields (20–30 % recoveries with 10 % acid).

When KCN solution was distilled with acid there was a 95–97 % recovery of the HCN. When benzaldehyde was added as well only 50–70 % was recovered.

The apparent reaction between benzaldehyde and HCN at the boiling point may explain the similar losses which occur during the distillation of the prulaurasin of cherry laurel and of amygdalin.

Aeration method.

The other method investigated was based on that of Roe [1924] for the estimation of amygdalin, where the glucoside is decomposed by means of emulsin and the HCN carried by a rapid current of air from the generating liquid into potash.

The method was tested with small amounts of amygdalin.

With 0·0400 g. of amygdalin in each experiment	With 0·00348 g. of amygdalin in each experiment
Recoveries	Recoveries
98·9 %	99·4 %
102 %	99·6 %
100·2 %	96·9 %
100·0 %	98·2 %

In applying the aeration method to leaves, steam was first used to inactivate the glucoside-hydrolysing enzyme, but it was found that nearly all the glucoside had been decomposed, as shown by the HCN liberated, before the inactivation occurred. Treub [1907] also found that he could not prevent the decomposition of less than $\frac{1}{2}$ to $\frac{3}{4}$ of the glucoside when suddenly heating leaves in steam.

The volatile product, HCN, here gives evidence of a great deal of enzyme action before the enzyme is inactivated. It suggests that in other cases, where the enzyme is supposedly inactivated instantaneously by plunging into boiling water or by steaming, a great deal of decomposition may occur before the inactivation takes place.

Plunging the leaf into boiling absolute alcohol was then tried, and even with this a part of the HCN is liberated (about 6 %). The cut leaves were dropped into the boiling alcohol in a flask, through which a current of air was passed into potash. The alcohol was distilled over into the potash, water and a small amount of emulsin were then added to the leaves, and the liberated HCN carried over into the potash in an air current. It was found that the alcohol had only a very slight effect on the end-point of the titration. This loss of HCN when leaves are dropped into boiling alcohol has also been shown by Treub [1907].

This "alcohol" method, although complicated, ensures that the action of other enzymes shall not interfere. The liberation of HCN at high temperatures and at the time of opening the flask is avoided. Hence the results were regarded as probably showing the whole of the HCN present as glucoside in the leaf.

After the alcohol method had been elaborated a simpler one for class experiments was devised in collaboration with the Hon. Mrs Onslow. In this the leaves were placed in cold water and gradually heated to 100° in a current of air. After cooling to 35° emulsin was added and the aeration carried on so that traces of glucoside remaining were decomposed.

Most of the glucoside is hydrolysed by the enzyme of the leaf while the temperature is being raised to 100°, but some still remains and is split by the added emulsin.

	(1)	(2)
HCN given off at the end of heating	95·5 %	91·3 %
HCN given off after emulsin addition	4·5 %	8·7 %

Owing to the slower heating the enzyme is inactivated at a temperature of 60–80° and the apparent loss of HCN which takes place in the distillation method does not take place here, since the results by this "water" method agree with those by the "alcohol" method, where nearly all (about 94 %) of the decomposition is by the added emulsin at 35°. The agreement between the two methods is shown by the following experiments with the corresponding halves of leaves.

In one experiment the results were 100 : 100·4 (HCN by "water" method: HCN by "alcohol" method), and in another 100 : 104·6. Since this was near

the limit of experimental error and within the variations between the corresponding halves of leaves, this was taken as showing that the simpler water method was about as accurate as the "alcohol" method. Hence the arguments on the non-interference of enzymes, etc. in the alcohol method must hold for the "water" method also. The simpler method was adopted in the final apparatus.

Estimation of the HCN. Since in the aeration method it is necessary to absorb the HCN in potash, titration in the alkaline solution by silver nitrate was the most convenient method. When the necessary precautions were taken this was found to be quite accurate enough for the present biological requirements. Results by this method were found to agree with those by the iodine method [Dunstan and Henry, 1904].

Using the blackened box and small volumes of titrated fluid the addition of one drop from the microburette (0·01 cc.) is sufficient to give a distinct end-point in clear solutions. Hence readings may be obtained accurate to 0·02 cc. of $M/100$ $AgNO_3$, which corresponds to 0·00001 g. of HCN. Thus the quantities of HCN in a single leaf, 0·001–0·005 g., can be estimated with sufficient accuracy.

It is difficult to obtain an idea of the error of the whole process when used with leaves, but with amygdalin in amounts corresponding to those of prulaurasin in a cherry laurel leaf, the error is 1–3 %. The HCN contents per 100 g. of fresh weight of the corresponding halves of leaves also agree within these limits.

It is of course open to question whether the HCN as measured in this way is representative of the cyanogenetic glucoside in the plant. This however seems probable, since in the "alcohol" method it is only the β-glucosidic HCN which can be liberated, as nearly all the decomposition is by added emulsin. Further, by the kindness of Mrs Onslow, I am able to state that the average of some of her unpublished determinations of the glucoside sugar of young cherry laurel leaves is in substantial agreement with the aeration results for corresponding leaves; moreover, data obtained during starvation by the two methods fall on closely parallel curves. The values for glucoside sugar give a somewhat lower amount of equivalent HCN. This is what would be expected since a certain amount of decomposition must have taken place, previous to the estimation, during the killing of the leaves in boiling absolute alcohol.

Summary.

Various methods of estimating the cyanogenetic glucosides in plant tissues are discussed and most of them considered unsatisfactory. In particular the distillation method has been found to give untrustworthy results.

Two new methods are described in which the HCN of the glucoside is liberated by enzyme action and carried over into potash in a current of air. The HCN absorbed is titrated in the alkaline liquid by silver nitrate. This method is considered satisfactory if certain details of procedure are adopted.

The "alcohol" method is considered to be a sound method for the estimation of cyanogenetic glucosides in leaves.

The results by this method agree with those by a simpler "water" method which was adopted for use in plant physiological investigation. The details of apparatus and procedure used in this are described.

It is hoped to publish elsewhere data obtained by this method on cherry laurel leaves.

This research was carried out during the tenure of a grant from the Department of Scientific and Industrial Research and later of the Frank Smart Studentship in Botany. I should like to tender my very grateful thanks to Dr F. F. Blackman, F.R.S., and to the Hon. Mrs Onslow for help and advice throughout the investigation.

REFERENCES.

Alsberg and Black (1916). *J. Biol. Chem.* **25**, 133.
Armstrong and Horton (1908). *Proc. Roy. Soc. Lond.* B, **80**, 321.
Dunstan and Henry (1904). *Proc. Roy. Soc. Lond.* **72**, 285.
Roe (1924). *J. Biol. Chem.* **58**, 667.
Treub (1907). *Ann. Jard. Bot. Buitenzorg*, **21**, 101.
Willaman (1917). *J. Biol. Chem.* **29**, 25.

CLVI. NEUTRAL SALT ADDITION COMPOUNDS OF ALKALINE EARTH GLUTAMATES AND ASPARTATES.

By WINSTON KENNAY ANSLOW and HAROLD KING.

From the Department of Biochemistry and Pharmacology, National Institute for Medical Research, Hampstead, N.W. 3.

(*Received September 1st, 1927.*)

It was shown a few years ago by King and Palmer [1920] that glycine could form a series of addition compounds with alkali halides and alkaline earth halides under experimental conditions free from dubiety, thus extending and confirming the earlier work of Pfeiffer and his co-workers. About the same time Hugounenq and Florence [1920], by the action of calcium carbonate upon glutamic acid hydrochloride, obtained an addition compound which they represented by either of the formulae

$$C_4H_8O_2N.CO_2CaCl.H_2O \text{ or } (C_4H_8O_2N.CO_2)_2Ca.CaCl_2.2H_2O,$$

but they were unable to isolate analogous compounds using barium or other metallic oxides. A year later [1921] the same observers prepared from the reaction product of aspartic acid hydrochloride and chalk, by alcoholic precipitation, a calcium chloride addition compound analogous to that from glutamic acid.

As no further work has appeared on these interesting products, which are essentially glycine derivatives with the added complication of an extra carboxyl group in salt formation, the subject has been examined more closely in the hope of deciding between the formulae advanced by Hugounenq and Florence and of arriving at some conclusion as to the mode of attachment of these inorganic addenda.

d-Glutamic acid was chosen as starting material since it is optically active and can be readily obtained in large quantities. In the experimental portion a process is described whereby 30·5 g. of pure *d*-glutamic acid hydrochloride may be obtained from 100 g. of gluten flour avoiding alcohol as a precipitant and also the laborious process of saturation with hydrogen chloride gas. In addition an effective modification of Hugounenq and Florence's method for preparing glutamic acid from the hydrochloride is described.

When glutamic acid hydrochloride, hydrobromide or hydriodide is boiled in aqueous solution with freshly precipitated calcium or barium carbonate or with the corresponding hydroxide followed by saturation with carbon

dioxide, the filtrates, on concentration, readily deposit well-formed crystals of the new addition compound of the type

$$(CO_2H.CHNH_2.CH_2.CH_2.CO_2)_2\,M.MA_2.xH_2O,$$

where M may be calcium or barium and A may be chlorine, bromine, or iodine.

In the case of strontium, the first crystalline crop was invariably strontium glutamate, so for purposes of comparison calcium and barium glutamates were prepared from free glutamic acid and found to be much more soluble than strontium glutamate. The exceptional behaviour of the strontium solutions is thus due to the solubility product of strontium glutamate being reached before that of the addition compounds. In confirmation of this it was found that if solutions of strontium glutamate were allowed to crystallise in presence of excess of strontium chloride, bromide or iodide, then addition compounds of the same type as those formed with calcium and barium halides were readily obtained.

In a similar manner aspartic acid hydrochloride and strontium carbonate gave the addition compound

$$(CO_2H.CHNH_2.CH_2.CO_2)_2\,Sr.SrCl_2.2H_2O$$

without the need for excess of strontium chloride, and definite evidence was also obtained for the formation of an addition compound between barium chloride and barium aspartate, but only in the presence of large excess of barium chloride. The calcium analogue obtained by Hugounenq and Florence by precipitation with alcohol could not be obtained by us, using aqueous media alone.

The chief interest of these compounds apart from their possible physiological significance lies in their constitution. The starting materials are optically active and a determination of the variation of specific rotation with concentration over a wide range of concentrations might be expected to cast some light upon their constitution. The results of such an experiment are shown graphically in Fig. 1, where a number of addition compounds are compared with the corresponding alkaline earth glutamates and alkali glutamates. The general trend of the curves is similar, from a region of positive rotation at high concentrations to a region of negative rotation at low concentrations, the whole corresponding to a change of molecular rotating species with dilution. At high dilutions, 1–2 %, the curves show a tendency to flatten out, and it is of interest that if the ionic value for the negative glutamate ion be calculated from these curves at a concentration in the region of 2·5 % then the ionic values show a measure of agreement.

Salt	$[\alpha]_{5461}$ for ion	Salt	$[\alpha]_{5461}$ for ion
$(C_5H_8O_4N)_2$ Ca.H_2O	−6·3°	$(C_5H_8O_4N)_2$ Ca.$CaCl_2.2H_2O$	−5·8°
$(C_5H_8O_4N)_2$ Ba.$5\frac{1}{2}H_2O$	−5·8°	$(C_5H_8O_4N)_2$ Ca.$CaBr_2.2H_2O$	−5·8°
$(C_5H_8O_4N)_2$ Sr.$4H_2O$	−6·1°	$(C_5H_8O_4N)_2$ Ba.$BaCl_2.6H_2O$	−6·3°
$C_5H_8O_4N$ Li	−5·9°	$(C_5H_8O_4N)_2$ Ba.$BaBr_2$	−6·2°
$C_5H_8O_4N$ Na	−5·5°	Mean	−6·0°

It seems justifiable to conclude that at high concentrations comparable indeed with saturated solutions, the non-ionised alkaline earth glutamates

and the non-dissociated additive compounds with alkaline earth halides are dextro-rotatory and possibly that the addition compounds do not markedly alter the constitution of the rotating unit.

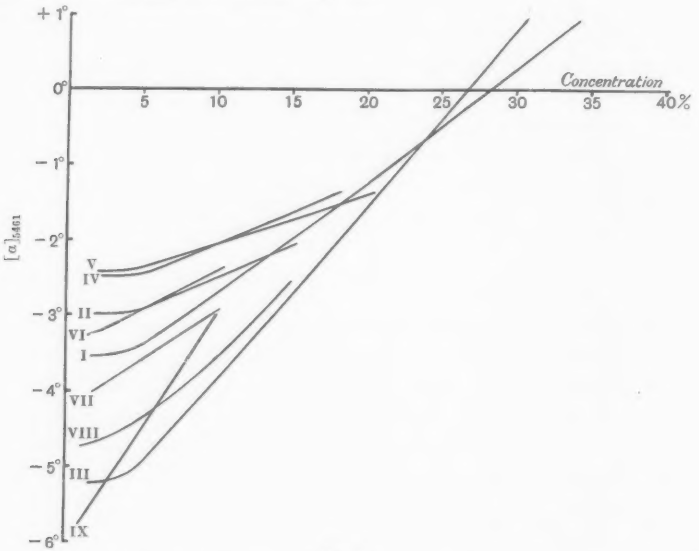


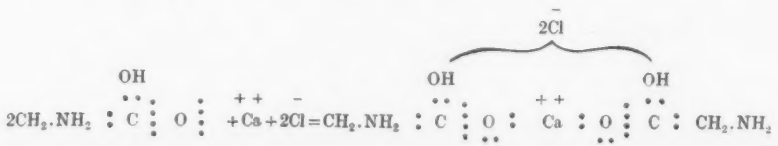
Fig. 1.

I = $(C_5H_8O_4N)_2Ca.CaCl_2.2H_2O$
II = $(C_5H_8O_4N)_2Ca.CaBr_2.2H_2O$
III = $(C_5H_8O_4N)_2Ca.H_2O$
IV = $(C_5H_8O_4N)_2Ba.BaCl_2.6H_2O$
V = $(C_5H_8O_4N)_2Ba.BaBr_2$
VI = $(C_5H_8O_4N)_2Ba.5\frac{1}{2}H_2O$
VII = $(C_5H_8O_4N)_2Sr.4H_2O$
VIII = $C_5H_8O_4NNa$
IX = $C_5H_8O_4NLi$

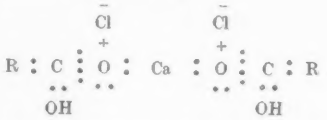
The constitution of the addition compounds of glycine and neutral salts has been ably discussed by Pfeiffer [1922] in terms of Werner's theory of residual affinity and co-ordination. There has however, as far as we are aware, been no discussion of these particular complexes from the point of view of electron sharing. The most frequent type of compound formed by glycine is

$$2CH_2NH_2.CO_2H.MA_2.xH_2O$$

where M is an alkaline earth and A is a halogen. This is readily interpreted electronically as follows, the indication of the electrons being here confined to atoms actually participating in the reaction

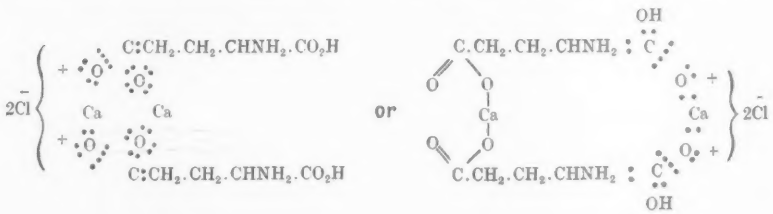


In the latter formula two electrons from each carbonyl oxygen atom enter the outer shell of the calcium atom, the oxygen atoms which were originally neutral thereby becoming positively charged and the calcium atom becoming doubly negatively charged. The calcium atom was however initially doubly positively charged through ionisation of the chlorine ions and the resultant neutralisation of charges on the calcium atom results in the chlorine ions being held to oxygen by oxonium valencies, thus



In these compounds therefore calcium has a co-ordination number of two.

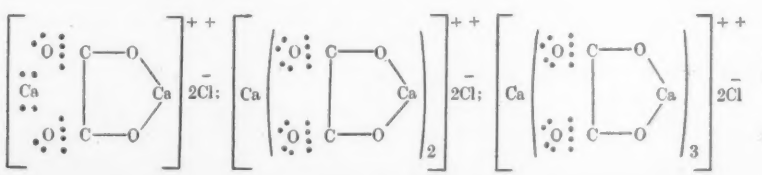
The alkaline earth glutamate and the aspartate addition compounds described in this paper are all of one type and fall into the same general scheme of electron sharing. Owing however to the presence of two carbonyl oxygen atoms in each glutamic or aspartic acid molecule there are two possible formulae and there is no evidence to decide between these. Thus the addition compound of calcium chloride and calcium glutamate can be written either as



Here again the neutralisation of charges results in the chlorine ions being held to oxygen by oxonium valencies. The view might be advanced that the chlorine ions are attached to the calcium by a co-valency, thus giving the calcium a co-ordination number of four, but the existence of calcium chloride addition compounds of calcium oxalate of the formulae

$$CaC_2O_4.CaCl_2.7H_2O,\ 2CaC_2O_4.CaCl_2.24H_2O \text{ and } 3CaC_2O_4.CaCl_2.8H_2O$$

makes such a supposition unlikely. To be consistent these are pictured as follows, the electrons being represented by dots



where the calcium has, progressively, co-ordination numbers of 2, 4 and 6 [cf. Pfeiffer, 1922]. If the chlorine atoms in the last of these were united by a co-valency, calcium would have the unlikely co-ordination number of eight.

Sugden [1927] has recently discussed molecular compounds in terms of singlet linkages but his views do not appear to present any advantage over the preceding.

Experimental.

Preparation of glutamic acid. Gluten flour[1] (100 g.) was added to warm concentrated HCl (300 cc., d. 1·16) and the mixture boiled for 6 hours. After treatment with charcoal and filtration the solution was kept below 0° for 12 hours and deposited 28·9 g. of crude glutamic acid hydrochloride. This crop was recrystallised from 50 cc. of boiling 20 % HCl and gave 23·7 g. of hydrochloride, which was dissolved in 20 cc. boiling water and treated with charcoal; it was then filtered and 33 cc. HCl (d. 1·16) were added. 21·6 g. of pure glutamic acid hydrochloride were obtained. By concentration of the original mother-liquors under reduced pressure further crops of crude glutamic acid hydrochloride mixed with ammonium chloride were obtained, and these recrystallised from the mother-liquors of the first crop gave finally a further 8·9 g. of pure glutamic acid hydrochloride. The total yield was therefore 30·5 g. from 100 g. of gluten. (Found: Cl, 19·3; N, 7·8. Theory: Cl, 19·3; N, 7·6%.) The rotation of the pure hydrochloride was determined at different concentrations with the following results:

c	10·0	7·5	5·0	2·5	1·25	0·6	0·5	0·25
$[\alpha]_{5461}$	+28·63°	+28·25°	+27·45°	+26·85°	+26·28°	+23·54°	+22·87°	+20·59°

The hydrochloride (36·7 g.) was dissolved in 200 cc. of water and heated on the water-bath. Aniline (18·6 g. = 1 mol.) was added and to the clear solution 200 cc. of 95 % alcohol. On cooling 27·55 g. of glutamic acid separated. It was recrystallised from the minimum volume (250 cc.) of boiling water and gave 24·1 g. of pure glutamic acid crystallising in hexagonal plates. From the mother-liquors a further 1·8 g. was obtained. (Found: N, 9·4. Theory: N, 9·5 %.)

Salts of glutamic acid. The hydrobromide, described by Hlasiwetz and Habermann [1873] as crystalline conglomerates, separates in large hexagonal tablets on treatment of glutamic acid with slight excess of 48 % HBr. Like the hydrochloride it melts at 214°, but this depends on the rate of heating. (Found: Br, 35·1. Theory: Br, 35·0 %.) The rotation was determined at different concentrations in water.

c	10·0	7·5	5·0	3·0	2·0	1·0	0·5
$[\alpha]_{5461}$	+23·09°	+23·30°	+23·13°	+22·50°	+22·0°	+20·57°	+18·70°

The hydriodide crystallises similarly on dissolving glutamic acid in slight excess of constant boiling HI freshly distilled over red phosphorus. It melts

[1] Purchasable from Messrs Bishop and Brooke, 21 Cock Lane, Snowhill, E.C.

with effervescence at 180–185°. (Found: I, 45·7; N, 5·1. Theory: I, 46·1; N, 5·1 %.) The only reference to this salt appears to be a crystallographic one by Kaplanova [1917].

The rotations of the salt were determined at various concentrations.

c	10·0	7·5	5·0	3·0	2·0	1·0	0·5
$[\alpha]_{5461}$	+18·64°	+18·39°	+18·35°	+18·14°	+17·59°	+15·79°	+11·57°

If the effect of variation of the concentration of the three halogen acid salts be plotted against specific rotation the curves shown in Fig. 2 are obtained.

There is a rapid falling off of specific rotation in solutions below 3 % due to hydrolysis of the salts, the values for dissociated salt approaching that of glutamic acid. Exact confirmation of this is found in the work of Wood [1914] who observed a similar phenomenon for several amino-acids when specific rotation was plotted against the ratio of the number of molecules of hydrochloric acid to molecules of amino-acid. If the specific rotation of the glutammonium ion be calculated from each salt at a dilution where hydrolysis is negligible there is substantial agreement between the observations.

Salt	$[\alpha]_{5461}$ ion
Glutamic acid hydrochloride	+33·4°
Glutamic acid hydrobromide	+34·6°
Glutamic acid hydriodide	+33·4°

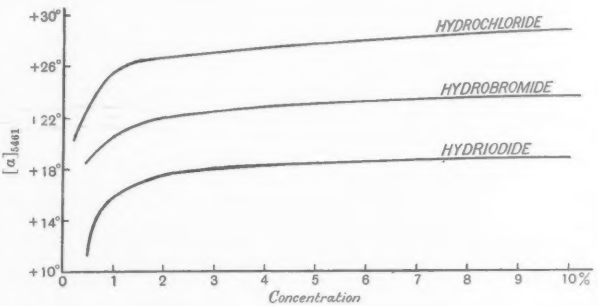


Fig. 2

Calcium glutamate. Prepared from pure glutamic acid and calcium carbonate, this salt crystallises slowly from syrupy solutions in small hexagonal plates which are readily soluble in water.

0·2000 g. dried at 160° lost 0·0073 g. H_2O = 3·7 %.
0·2052 g. dried at 160° lost 0·0087 g. H_2O = 4·2 %.
0·1927 g. dry; NH_3 = 12·01 cc. $N/10$ acid. N = 8·7 %.
0·1783 g. dry; 0·0751 g. $CaSO_4$. Ca = 12·4 %.

Calc. for $(C_5H_8O_4N)_2Ca.H_2O$; H_2O = 5·1 %: for $(C_5H_8O_4N)_2Ca$; Ca = 12·1 %; N = 8·4 %.

The rotations were determined over a wide range of concentrations.

c	30·0	25·0	20·0	15·0	10·0	5·0	2·5
$[\alpha]_{5461}$	+0·81°	−0·34°	−1·71°	−2·62°	−3·84°	−5·05°	−5·22°

Strontium glutamate. This salt crystallises very readily from concentrated solutions and separates in hexagonal plates.

0·2105 g. dried at 95° lost 0·0353 g. $H_2O = 16·8$ %.
0·2945 g.; 0·1152 g. $SrSO_4$. Sr = 18·7 %.
0·1752 g. dry; $NH_3 = 8·85$ cc. $N/10$ acid. N = 7·1 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.$4H_2O$; Sr = 19·3 %; $H_2O = 16·0$ %: for $(C_5H_8O_4N)_2$ Sr; N = 7·4 %.

It gave the following specific rotations:

c	10·0	5·0	2·5
$[\alpha]_{5461}$	−2·96°	−3·59°	−3·95°

Barium glutamate. This salt crystallises in irregular hexagonal plates from concentrated solutions.

0·2093 g. dried at 160° lost 0·0376 g. $H_2O = 18·0$ %.
0·2040 g.; $NH_3 = 7·87$ cc. $N/10$ acid. N = 5·4 %.
0·1975 g.; 0·0881 g. $BaSO_4$. Ba = 26·2 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$5\frac{1}{2}H_2O$; $H_2O = 18·0$ %; N = 5·3 %; Ba = 26·2 %.

The following specific rotations were obtained:

c	10·0	5·0	2·5
$[\alpha]_{5461}$	−2·44°	−2·94°	−3·22°

Lithium glutamate. This salt crystallises in irregular hexagonal plates which are anhydrous.

0·1959 g.; $NH_3 = 12·43$ cc. $N/10$ acid. N = 8·9 %.
Calc. for $C_5H_8O_4N$ Li. N = 9·2 %.

The specific rotations of this salt were as follows:

c	10·0	5·0	2·5	1·25
$[\alpha]_{5461}$	−3·01°	−4·52°	−5·19°	−5·68°

Sodium glutamate. A standard solution was made up from a known weight of pure glutamic acid and standard alkali. The specific rotations were determined at a variety of concentrations.

c	15·0	10·0	5·0	2·5	1·25
$[\alpha]_{5461}$	−2·57°	−3·57°	−4·30°	−4·67°	−4·75°

Addition compounds of alkaline earth glutamates.

Calcium glutamate calcium chloride. Glutamic acid hydrochloride (9·2 g.) was boiled with 50 cc. water and excess of calcium carbonate; the solution was filtered and concentrated. On keeping, the dihydrate of calcium glutamate calcium chloride (5·8 g.) separated in hexagonal plates. It also crystallises in needles. To avoid the known decomposing action of water on certain addition compounds, the crystalline crops were in every case filtered on hardened paper without washing and then rubbed dry on porous plate before analysis.

0·1138 g. dried at 160° lost 0·0086 g. $H_2O = 7·6$ %.
0·2046 g.; 0·1211 g. AgCl. Cl = 14·7 %.
0·2062 g.; $NH_3 = 8·7$ cc. $N/10$ acid. N = 6·0 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaCl_2$.$2H_2O$; Cl = 14·8; N = 5·8; $H_2O = 7·5$ %.

It was unchanged by recrystallisation from water in which it is readily soluble (1 in 2·5 parts).

0·2024 g.; 0·1191 g. AgCl. Cl = 14·6 %.

On recrystallisation from solutions containing 2 or 5 molecules excess of calcium chloride, the same compound was obtained in each case.

2 molecules excess—0·1886 g. (anhydrous); 0·1265 g. AgCl. Cl = 16·6 %.
5 molecules excess—0·1746 g. (anhydrous); 0·1206 g. AgCl. Cl = 17·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaCl_2$; Cl = 16·0 %.

The specific rotations of solutions of the addition compound at a variety of concentrations were determined with the following results:

c	33·8	26·9	21·0	14·9	10·0	5·0	3·0	2·0	1·3
$[\alpha]_{5461}$	+0·9°	−0·18°	−1·13°	−2·02°	−2·7°	−3·40°	−3·50°	3·38° −	−3·56°

Calcium glutamate calcium bromide. Glutamic acid hydrobromide (11·4 g.) was boiled with excess of calcium carbonate in 50 cc. water; the solution was filtered and concentrated. The addition compound separated on keeping in hexagonal plates (6·9 g.).

0·2060 g. dried at 160° lost 0·0145 g. H_2O = 7·0 %.
0·1917 g.; 0·1255 g. AgBr. Br = 27·9 %.
0·1927 g.; NH_3 = 7·06 cc. *N*/10 acid. N = 5·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaBr_2.2H_2O$; Br = 28·0 %; N = 4·9 %; H_2O = 6·3 %.

The addition compound gave the following specific rotations:

c	15·0	10·0	5·0	3·75	1·9
$[\alpha]_{5461}$	−2·07°	−2·45°	−2·95°	−3·0°	−3·0°

Calcium glutamate calcium iodide. Prepared similarly to the preceding, this addition compound crystallised in irregular hexagonal plates.

0·1997 g. dried at 160° lost 0·0140 g. H_2O = 7·0 %.
0·1997 g.; 0·1380 g. AgI. I = 37·8 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaI_2.2\frac{1}{2}H_2O$; I = 37·8 %; H_2O = 6·7 %.

Barium glutamate barium chloride. Glutamic acid hydrochloride (9·2 g.) was dissolved in water, made alkaline with baryta and the boiling solution was saturated with carbon dioxide. On filtration and concentration the addition compound (11·3 g.) separated in irregular hexagonal plates.

0·2020 g. dried at 160° lost 0·0299 g. H_2O = 14·8 %.
0·1721 g. (anhydrous); 0·0794 g. AgCl. Cl = 11·4 %.
0·1712 g. (anhydrous); NH_3 = 5·91 cc. *N*/10 acid. N = 4·8 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaCl_2$; Cl = 11·1 %; N = 4·4 %: for $(C_5H_8O_4N)_2$ Ba.$BaCl_2.6H_2O$; H_2O = 14·5 %.

The following specific rotations were obtained at various concentrations:

c	18·0	15·0	10·0	5·0	4·0	3·0	2·0
$[\alpha]_{5461}$	−1·38°	−1·62°	−1·90°	−2·45°	−2·49°	−2·25°	−2·47°

Barium glutamate barium bromide. Prepared similarly to the above, glutamic acid hydrobromide (11·4 g.) gave 8·4 g. of the addition compound crystallising in minute hexagonal plates which were *anhydrous*.

0·1896 g.; 0·0963 g. AgBr. Br = 21·6 %.
0·2018 g.; NH_3 = 5·9 cc. *N*/10 acid. N = 4·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaBr_2$; Br = 22·0 %; N = 3·9 %.

On recrystallisation from water a *tetrahydrate* was obtained.

0·2040 g. dried at 160° lost 0·0191 g. H_2O = 9·4 %.
0·1894 g. (anhydrous); 0·0942 g. AgBr. Br = 21·7 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaBr_2.4H_2O$; H_2O = 9·0 %.

The specific rotations of the anhydrous salt at various concentrations were as follows:

c	20·0	15·0	10·0	5·0	4·0	3·0
$[\alpha]_{5461}$	−1·41°	−1·74°	−2·03°	−2·38°	−2·34°	−2·49°

Barium glutamate barium iodide. Prepared similarly to the preceding, this addition compound separated in irregularly shaped crystals.

0·1938 g. dried at 120° lost 0·0252 g. $H_2O = 11·4$ %.
0·1686 g. (anhydrous); 0·0977 g. AgI. I = 31·3 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.BaI_2; I = 30·9 %: for $(C_5H_8O_4N)_2$ Ba.BaI_2.$6H_2O$; $H_2O = 11·6$ %.

Strontium glutamate strontium chloride. Glutamic acid hydrochloride (9·2 g.) was boiled in 50 cc. water with excess of strontium carbonate; the solution was filtered and concentrated. On keeping, a large crop (6·9 g.) separated of homogeneous appearance and consisting of microscopic narrow leaflets. It proved on analysis to be mainly strontium glutamate. (Found: Cl = 1·2 %.) The mother-liquors on concentration deposited tablets (1·8 g.) which were evidently crude addition compound (Cl = 9·6; N = 3·6 %, whence Cl : N = 1·07 : 1). On further concentration the liquors gave crude strontium chloride (1·7 g.) crystallising in long needles. (Found: Cl = 36·6 %.)

The first crop on recrystallisation from 12 cc. of boiling water gave pure strontium glutamate crystallising in rectangular plates.

0·2042 g. dried at 95° lost 0·0350 g. $H_2O = 17·1$ %.
0·1692 g. (anhydrous); NH_3 equivalent to 8·84 cc. $N/10$ acid. N = 7·3 %.
Calc. for $(C_5H_8O_4N)_2$ Sr; N = 7·4 %: for $(C_5H_8O_4N)_2$ Sr. $4\frac{1}{2}H_2O$; $H_2O = 17·1$ %.

The pure addition compound was obtained as follows. Glutamic acid hydrochloride (4·6 g.) was treated with 50 cc. N hydrochloric acid, made alkaline by addition of strontia and the boiling solution was saturated with carbon dioxide. On filtration and concentration the addition compound separated in bold tablets. Preliminary essays showed that even with this excess of strontium chloride, strontium glutamate tended to separate first.

0·2047 g. dried at 160° lost 0·0642 g. $H_2O = 31·4$ %.
0·2047 g.; 0·0802 g. AgCl. Cl = 9·7 %.
0·2102 g.; $NH_3 = 5·84$ cc. $N/10$ acid. N = 3·9 %.
0·1965 g.; 0·0980 g. $SrSO_4$. Sr = 23·8 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.$SrCl_2$.$12H_2O$; Cl = 9·4 %; N = 3·7 %; Sr = 23·2 %; $H_2O = 28·7$ %.

Strontium glutamate strontium bromide. Glutamic acid hydrobromide (2·8 g.) was treated in aqueous solution with excess of strontia and the boiling solution saturated with carbon dioxide. On filtration and concentration microscopic needles (2·1 g.) of crude strontium glutamate (found: N, 5·5 %; Br, 3·3 %) separated. The mother-liquors, now relatively richer in strontium bromide, deposited on concentration a homogeneous crop of needles, quite different in appearance from the first crop, which proved to be the required addition compound.

0·2020 g.; 0·0919 g. AgBr. Br = 19·4 %.
0·2123 g.; $NH_3 = 4·7$ cc. $N/10$ acid. N = 3·1 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.$SrBr_2$.$11H_2O$; Br = 19·4 %; N = 3·4 %.

Strontium glutamate strontium iodide. A solution made up in the usual way from 3·4 g. glutamic acid hydriodide deposited 2·35 g. crude strontium

glutamate (found: I = 3·9 %; N = 5·5 %). The mother-liquors, now relatively richer in strontium iodide deposited on further concentration well-formed hexagonal plates (0·55 g.) of the required addition compound.

0·1560 g.; 0·0863 g. AgI. I = 29·9 %.
0·2215 g.; NH_3 = 5·00 cc. *N*/10 acid. N = 3·2 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.SrI_2.$7H_2O$; I = 29·9 %; N = 3·3 %.

Lithium glutamate and lithium halides. From solutions of glutamic acid hydrochloride, hydrobromide or hydriodide and lithium carbonate only crude lithium glutamate was isolated (found in the three cases respectively: Cl = 1·2 %; Br = 1·9 %; I = 3·9 %).

Calcium chloride and glutamic acid hydrochloride. Solutions of glutamic acid hydrochloride containing 2 or 5 molecular proportions of calcium chloride deposited crude glutamic acid hydrochloride. (Found: Cl = 22·0 %; 23·9 % respectively.)

Calcium chloride and glutamic acid. When glutamic acid was crystallised from a solution containing two molecular proportions of calcium chloride it was recovered contaminated with a small proportion of chloride. (Found: Cl = 2·0 %.)

Addition compounds of alkaline earth aspartates.

Strontium aspartate strontium chloride. Aspartic acid (2·66 g.), prepared from asparagine, was dissolved in 20 cc. hot *N* hydrochloric acid (1 mol.) and boiled gently with excess of strontium carbonate. On filtration and slight concentration the solute crystallised readily as a micro-crystalline powder (1·85 g.).

0·2110 g. dried at 160° lost 0·0128 g. H_2O = 6·1 %.
0·1990 g.; 0·1346 g. $SrSO_4$. Sr = 32·3 %.
0·2011 g.; 0·0975 g. AgCl. Cl = 12·3 %.
0·1937 g.; NH_3 = 6·84 cc. *N*/10 acid. N = 4·9 %.
Calc. for $(C_4H_6O_4N)_2$ Sr.$SrCl_2$.$2H_2O$; Sr = 32·1 %; Cl = 13·0 %; N = 5·1 %; H_2O = 6·6 %.

On concentration the mother-liquors deposited the same addition compound (1·25 g.).

Barium aspartate and barium chloride. Aspartic acid (1·33 g.) was treated with 100 cc. 0·1 *N* hydrochloric acid (1 mol.), made alkaline with baryta and then carbon dioxide was passed into the boiling solution in excess. The filtered solution was concentrated to about 10 cc. and the compound crystallised in microscopic narrow leaflets of homogeneous appearance, yield 1·7 g. On analysis it gave Ba = 35·8 %, Cl = 5·0 %; N = 5·2 %, corresponding to a mixed crystal containing barium chloride and barium aspartate in the ratio 1 : 2·6 respectively.

When a similar quantity of aspartic acid was subjected to the same series of operations but using three molecular proportions of hydrochloric acid, the crystalline product consisted of microscopic rhomb-shaped plates, yield 1·7 g. On analysis it gave Ba = 39·6 %, Cl = 7·6 %, N = 5·2 %, corresponding to

a mixed crystal, this time relatively richer in barium chloride, containing barium chloride and barium aspartate in the ratio 1: 1·7 respectively.

Calcium aspartate and calcium chloride. No crystalline product could be obtained from equimolecular proportions of these compounds in water.

REFERENCES.

Hlasiwetz and Habermann (1873). *Liebig's Ann.* **169**, 158.
Hugounenq and Florence (1920). *Bull. Soc. Chim.* **27**, 750.
—— —— (1921). *Bull. Soc. Chim. Biol.* **3**, 283.
Kaplanova (1917). *Jahr. Min.* **1**, 123.
King and Palmer (1920). *Biochem. J.* **14**, 574.
Pfeiffer (1922). Organische Molekülverbindungen (Stuttgart).
Sugden (1927). *J. Chem. Soc.* 1181.
Wood (1914). *J. Chem. Soc.* **105**, 1991.

CLVII. THE PHOSPHORUS OF CASEINOGEN.

I. ISOLATION OF A PHOSPHORUS-CONTAINING PEPTONE FROM TRYPTIC DIGESTS OF CASEINOGEN.

By CLAUDE RIMINGTON.

(*Benn W. Levy Student, Research Scholar of Emmanuel College, Cambridge.*)

From the Biochemical Laboratory, Cambridge.

(*Received August 29th, 1927.*)

THE work described in this paper was commenced in 1924, and a preliminary report issued in the following year [Rimington and Kay, 1925], in which it was stated that from tryptic digests of caseinogen a preparation had been obtained containing amino-acids in association with phosphoric acid, and representing approximately 10 % of the organic phosphorus of the original digest.

Since that time, experimental work has been directed along two main lines of approach. In the first place an investigation of the behaviour of caseinogen towards various proteolytic enzymes, phosphatases, acids and alkalis was made [Rimington and Kay, 1926; Rimington, 1927, 1], from which it was possible to draw certain conclusions relative to the state of combination of phosphorus in the caseinogen molecule. The linkage involved appears to be of the ester type, phosphoric acid being united to some hydroxyl-containing constituents of the protein molecule, most probably hydroxyamino-acids. A study of artificially phosphorised proteins [Rimington, 1927, 1] afforded additional evidence in support of this view.

At the same time experiments were being continued having as their object the isolation in a pure state of the phosphorus-containing substance present in tryptic digests of caseinogen. This has now been accomplished, and it is with this isolation that the present paper is concerned.

ISOLATION OF A PHOSPHORUS-CONTAINING PEPTONE.

The liberation from caseinogen, during the action of trypsin, of an acid-soluble, organic substance containing all the phosphorus of the protein has been noted by Biffi [1898], Plimmer and Bayliss [1906] and others, but neither Dr Kay nor myself was aware, when these experiments were begun, that any serious attempt had been made to isolate this substance. Our attention has since been called by Posternak [1927] to a Swiss patent taken out by

the Society of Chemical Industry in Basle [1923] in which the separation of a preparation containing organically combined phosphorus from caseinogen digests is described. A discussion of this and of Posternak's later work will be found below.

In an earlier communication it was shown that the liberation of the soluble organic substance by trypsin occurs with great rapidity, the subsequent splitting off of phosphoric acid taking place at a much slower rate. Isolation at the period when the soluble organic phosphorus was at a maximum did not however yield the best results as the quantity of peptide material present in these early stages was relatively great. By trial it was found that the best time to commence operations was 38–40 hours after the addition of trypsin. In illustration of the process, the details of an actual preparation are given.

1 kg. of caseinogen was digested with 500 cc. strained extract of pancreas in the presence of toluene for 38 hours at 37° and a p_H of 8·4 to 8·6, the total volume of liquid being 11 l. Glacial acetic acid was then added to bring the p_H to 4·5 and the whole filtered through fine linen. The filtrate (11·1 l.) contained 4·2 g. organic phosphorus.

1·8 l. of saturated lead acetate and 700 cc. of ammonia were then added in the order mentioned, the supernatant liquid removed and the precipitate filtered off. After repeated washing with water, it was suspended in water and decomposed by hydrogen sulphide. The filtrate, freed from hydrogen sulphide by a stream of air, was rendered alkaline with ammonia and magnesia mixture added with vigorous stirring. Precipitated magnesium ammonium phosphate was filtered off.

The filtrate was now adjusted to p_H 3·8 by means of acetic acid and hot saturated uranium acetate solution added until no further precipitation occurred. The precipitate was filtered off, washed thoroughly, and suspended in water, to this suspension sufficient 10 % sulphuric acid being added to effect complete solution. Well cooled 40 % sodium hydroxide was then added with vigorous stirring until all the uranium was precipitated. This was then filtered off by suction into flasks containing a little acetic acid in order to neutralise the filtrate immediately, since the substances sought are unstable in alkaline solution. No other method of removing the uranium was found to be practicable, but it was possible to carry out the whole of the above process, from the addition of the sodium hydroxide to the collection of the filtrates, within 5 minutes.

The uranium-free filtrates were combined, the resulting liquid having a slightly acid reaction. It possessed an N/P ratio of approximately 5/1, gave a strong biuret reaction (red) but no Millon and no glyoxylic reactions. A solution of phosphotungstic acid produced no precipitate.

The insoluble copper salt was next prepared by the addition to the solution of sufficient saturated copper acetate to produce complete precipitation. About 2 l. of copper acetate solution were required, the total volume amounting to 5 l.

This precipitate was filtered off, washed and dried over sulphuric acid *in vacuo* (copper precipitate I): weight 55·5 g.; total phosphorus 4·81 %; total nitrogen 7·50 %; ratio N/P = 3·45: yield 65 % of the organic phosphorus of the digest. For this stage the above yield is typical, although on one occasion as much as 74 % of the original organic phosphorus of the digest was obtained.

This substance, whose ratio N/P did not differ greatly from one of the preparations described by Posternak [1927], was not yet pure as was shown by fractional precipitation with copper acetate in the following manner.

15 g. copper precipitate I, N/P = 3·45, were suspended in water and decomposed by hydrogen sulphide. After filtration, using well washed "fuller's earth[1]" to remove the last traces of colloidal copper sulphide, the liquid was again treated with saturated copper acetate solution, 280 cc. being required to produce complete precipitation. The resulting precipitate (copper precipitate II) was separated, washed and decomposed as before. The solution was now treated with copper acetate in successive small quantities, the precipitates produced at each addition being separately removed. A fractionation was thus achieved, the four precipitates, referred to as copper precipitate III *A*, *B*, *C* and *D* respectively, being analysed for phosphorus and nitrogen with the following results.

Table I.

Cu ppt. III	Weight g.	Phosphorus %	Nitrogen %	N/P
A	1·72	6·34	7·44	$\frac{2·60}{1}$
B	5·50	5·50	7·32	$\frac{2·95}{1}$
C	2·53	5·32	7·43	$\frac{3·09}{1}$
D	2·22	5·09	7·30	$\frac{3·18}{1}$
Total recovered	11·97			

It is evident that the greater part of the material, amounting to 67 % of the total quantity, comes down in the middle fractions (*B* and *C*) with a ratio N/P = 3. Repeated reprecipitation and fractionation of this material failed to alter its N/P ratio. It was therefore considered to be homogeneous and analysed[2] with the following result, from which the empirical formula $(C_{37}H_{53}O_{33}N_9P_3)Cu_{4·5}$ was deduced. It contained no sulphur.

	C	H	O	N	P	Cu
Calculated	28·98	3·62	34·44	8·22	6·07	18·66
Found (mean of duplicate analyses)	28·63	4·18	34·68	7·84	6·05	18·62

[1] "Fuller's earth" proved to be a very effective adsorbent for colloidal copper sulphide but to retain not more than traces of the phosphorus-containing substances.

[2] The analysis recorded in this and the following paper [1927] have been carried out either by Mr F. Flack of the Chemical Laboratory, or by Mr A. R. Colwell, of the Biochemical Laboratory, Cambridge or by "Fein Chemie," Tübingen.

The purified substance represented just under 50 % of the original organic phosphorus of the caseinogen digest. It was a light blue powder, insoluble in water but yielding solutions, when the copper was removed by hydrogen sulphide, which gave an intense red biuret reaction. The preparation of the free acid in the dry state is described below.

The two fractions *A* and *D*, obtained during the purification of the crude material, were investigated in order to ascertain whether they contained other peptones in addition to that described above. The material *A* had a ratio N/P = 2·6 not far removed from that required by a substance containing 8 nitrogen atoms to 3 of phosphorus. Upon analysis, however, it was found to be a mixture of the peptone already described and copper phosphate. The fraction *D* had a ratio N/P = 3·18, whilst that of the filtrate from the precipitation was 3·4, indicating that whatever impurity is present at this stage is there only in small quantity. All attempts to isolate from these fractions pure material having a higher ratio of nitrogen to phosphorus than that of the peptone described were without success. There appears to be some phosphorus-free material which follows the precipitation of the phosphorus-containing peptone rather closely, and it seems possible that this may account for the variety of preparations with varying N/P ratios found by Posternak [1927].

Preparation and properties of the free phosphopeptone.

15 g. pure copper salt were decomposed by hydrogen sulphide and the resulting solution concentrated over sulphuric acid *in vacuo* at room temperature. There resulted a highly viscous syrup which solidified to a "glass." All efforts to obtain crystalline material were unsuccessful. Upon dehydration with absolute alcohol and pulverisation the peptone was obtained as a white, slightly hygroscopic powder, dissolving readily in water to yield distinctly acid solutions. Difficulty was experienced in effecting the combustion of this material, but on analysis it gave the following figures, which agree fairly well with the formula $C_{37}H_{62}O_{33}N_9P_3$ calculated from the composition of the copper salt.

	C	H	N	P
Found	37·44	5·75	10·13	7·05
Calculated	35·38	5·14	10·03	7·41

The amino-nitrogen, determined by the method of Van Slyke, was 1/9 of the total nitrogen; after hydrolysis with hydrochloric acid, however, the amino-nitrogen became equal to the total nitrogen. One would infer therefore that the substance is a peptone consisting of 9 amino-acids in peptide linkage, all of which are acyclic mono-amino-acids. An aqueous solution gave the following colour and precipitation tests:

Ninhydrin	+	Picric acid	–
Biuret	+ (deep rose)	Phosphotungstic acid	–
		Trichloroacetic acid	–
Glyoxylic	–	Lead acetate	dense white precipitate
Millon	–	Uranium acetate	voluminous yellow precipitate
Xanthoproteic	–	Copper acetate	blue precipitate
Sulphur test	–	Mercuric acetate	dense white precipitate
Molisch	–	Silver nitrate + ammonia	gelatinous white precipitate

It was noted in addition that its aqueous solutions gave a deep, orange-red colour with diazobenzenesulphonic acid and sodium carbonate, which turned golden yellow on addition of hydrochloric acid but left no secondary colour whatever after reduction with zinc dust and hydrochloric acid and addition of ammonia. The diazo-reaction could not therefore be due to histidine, which gives a yellow secondary colour; it was afterwards traced to hydroxyglutamic acid which is present in the peptone and which, as observed by Dakin [1918], yields the primary colours described. An investigation of the products of hydrolysis is presented in the following paper.

The colour tests demonstrate the absence of tryptophan, tyrosine, aromatic substances, carbohydrates, cystine and histidine. The high oxygen content on the other hand suggests that hydroxyamino-acids may be present together with phosphoric acid.

As has been previously reported [Rimington and Kay, 1926], investigation showed that the substance was slowly hydrolysed by pancreatic juice, phosphoric acid being produced and the peptide linkages also attacked. Phosphatases on the other hand bring about the separation of the phosphorus as phosphoric acid within a few hours. Sodium hydroxide (0·25 *N*) also liberates the whole of the phosphorus in inorganic form in 24 hours at 37°. Phosphopeptone is freely diffusible through collodion sacs.

Molecular weight. The determination of the molecular weight of the peptone could not be accomplished by any of the standard physico-chemical methods owing to its high degree of ionisation in solution, results being obtained which were very much too low. No better success was obtained when using the barium salt. The insolubility of the substance in camphor precludes employment of the method of Rast [1922]. From the analytical data, however, the ratio of amino-nitrogen to total nitrogen and from the action of bone phosphatase, which only removes 2/3 of the total phosphorus as phosphoric acid, it seems that the formulation as given is most likely correct. In view of the ready diffusibility of the substance it is assumed that the molecular weight is that indicated by the empirical formula, namely, 1254.

Optical activity. The peptone is strongly laevo-rotatory. The light used was the mercury green.

α in 2 dm. tube $-$ 2·659°.

Nitrogen determination on solution 165·6 mg. per 100 cc.

$$[\alpha]^{15°}_{5461} = -80{\cdot}53°,$$

$$M[\alpha] = -1011{\cdot}53.$$

Basicity. The basicity was determined by titration, using 0·1 *N* sodium hydroxide and thymolphthalein as indicator.

0·1 g. free acid required 6·9 cc. sodium hydroxide, whence basicity = 8·7.

The result is in good agreement with the analysis of the copper and barium salts which show the peptone to behave as a nine-basic acid.

Brucine salt. It was hoped that the peptone might yield a crystalline salt with one of the alkaloids. To a strong aqueous solution of the free peptone, nine equivalents of brucine were added together with a little methyl alcohol to aid solution. The resulting solution was neutral in reaction. A slight excess of brucine was then added so that the mixture was alkaline to phenol red. No crystalline material separated, although within the course of a few hours an oil had precipitated. This was separated off, and allowed to stand for several months. It could not, however, be induced to crystallise but gave a glassy material which was ground, very thoroughly extracted with absolute alcohol, and the resulting powder dried *in vacuo* for analysis.

	N	P
Found	8·00	2·10
Calc. for $C_{244}H_{286}O_{64}N_{27}P_2$	8·02	1·98

It appears that the substance was the brucine salt of the peptone containing nine equivalents of brucine, with a molecular weight of approximately 4700.

Barium salt. The barium salt was prepared by adding a saturated solution of baryta to a concentrated aqueous solution of the peptone until faintly alkaline and then pouring into alcohol. The insoluble barium salt was centrifuged off, washed repeatedly with absolute alcohol and dried *in vacuo*. It formed a fine white powder very soluble in water.

Analysis $(C_{37}H_{53}O_{23}N_9P_3)Ba_{4·5}$.

	N	P	Ba
Found	6·25	4·60	32·26
Calculated	6·76	4·99	33·14

Discussion.

Since the proof by Hammarsten [1877] that phosphorus is an integral constituent of the caseinogen molecule, research has repeatedly been directed to the discovery of the type of linkage by which this element is held in combination. By analogy with sulphur, it might be supposed that there exists a phosphorus-containing amino-acid; however, the ready production of orthophosphoric acid from caseinogen under the action of various mild reagents has led to the view that it is phosphoric acid which is built in some way into the nitrogenous complex.

Among the early attempts to separate phosphorus-containing material from caseinogen those of Salkowski [1901], Levene and Alsberg [1901] and Reh [1907] may be mentioned. All of these workers however used as their starting material peptic digests in which an enormous quantity of peptide material was present. In no case was any substance isolated in a pure form. Posternak [1927] has lately reported the isolation of a variety of phosphorus-containing substances from tryptic digests of caseinogen. His experimental methods are not cited, nor are analytical data presented, so that it is difficult to form any opinion of his work. In the experience of the present writer material has several times been isolated which bore a close resemblance, from

the point of view of elementary composition, to some of the preparations described by Posternak; in all cases however further work showed that these substances were still impure and that by suitable manipulation their components could be separated. In view of the above findings I find it difficult to believe that Posternak's preparations were homogeneous, and moreover I have been unable to confirm his statements regarding the structural units with which phosphoric acid is associated (see following paper). The alternative remains however, that the methods employed by him may have been so different from mine as to lead to dissimilar products. Of this, for the reasons stated above, I am unable to judge.

The phosphorus-containing complex present in tryptic digests of caseinogen appears to be largely, if not entirely, a phosphorus-containing peptone in which the phosphorus is present as phosphoric acid united by ester linkages to some or other of the amino-acids. The yield in which this purified peptone may be isolated corresponds to about 50 % of the original organic phosphorus of the digest. Although not entirely trypsin-stable, its peptide linkages are comparatively resistant to this enzyme; the phosphorus is also liberated with great slowness by trypsin. Phosphatases, on the other hand [Rimington and Kay, 1926], bring about a very rapid liberation of the phosphorus as phosphoric acid.

Summary.

1. A phosphorus-containing peptone, $C_{37}H_{62}O_{33}N_9P_3$, has been isolated from tryptic digests of caseinogen in a yield corresponding to 50 % of the organic phosphorus of the digest.

2. It is a strongly acidic substance forming well-defined copper and barium salts each containing nine equivalents of the metal. It titrates as a nine-basic acid. A brucine salt has also been obtained. The free acid has $[\alpha]_{5461}^{15°} = -80{\cdot}53°$.

3. The substance, which has been designated "*phosphopeptone*," contains 1/9 of its nitrogen in the form of amino-nitrogen. After hydrolysis the ratio amino-nitrogen/total nitrogen becomes unity.

4. Colour tests indicate the absence of the usual amino-acids, the biuret and ninhydrin reactions are however positive. A primary colour reaction is also given with diazobenzenesulphonic acid, but there is no secondary colour after reduction.

5. The peptide linkages of phosphopeptone are slowly attacked by trypsin, which also brings about the slow liberation of the phosphorus as phosphoric acid.

In conclusion I wish to express my thanks to Sir F. G. Hopkins for the interest he has taken in this work. My best thanks are also due to Dr H. D. Kay for much valuable criticism and many helpful suggestions.

REFERENCES.

Biffi (1898). *Virchow's Arch.* **152**, 130.
Dakin (1918). *Biochem. J.* **12**, 290.
Hammarsten (1877). Zur Kenntn. d. Caseins, etc. (Upsala).
Levene and Alsberg (1901). *Z. physiol. Chem.* **31**, 543.
Plimmer and Bayliss (1906). *J. Physiol.* **33**, 439.
Posternak (1927). *Compt. Rend. Acad. Sci.* **184**, 306; *Biochem. J.* **21**, 289.
Rast (1922). *Ber. deutsch. chem. Ges.* **55**, 1051.
Reh (1907). *Hofmeister's Beitr.* **11**, 1.
Rimington (1927, 1). *Biochem. J.* **21**, 204.
—— —— (1927, 2). *Biochem. J.* **21**, 272.
—— —— (1927, 3). *Biochem. J.* **21**, 1187.
Rimington and Kay (1925). *Chem. Ind.* **44**, 256.
—— —— (1926). *Biochem. J.* **20**, 777.
Salkowski (1901). *Z. physiol. Chem.* **32**, 245.
Society of Chemical Industry, Basle (1923). Swiss patent, No. 104336.

CLVIII. THE PHOSPHORUS OF CASEINOGEN.

II. CONSTITUTION OF PHOSPHOPEPTONE.

By CLAUDE RIMINGTON.

(*Benn W. Levy Student, Research Scholar of Emmanuel College, Cambridge.*)

From the Biochemical Laboratory, Cambridge.

(*Received August 29th, 1927.*)

In the preceding paper [Rimington, 1927, 2] the isolation from caseinogen of a pure phosphorus-containing peptone has been described. This substance, which has been named *phosphopeptone*, and whose composition is represented by the formula $C_{37}H_{62}O_{33}N_9P_3$, has been isolated in a yield corresponding to 50 % of the organic phosphorus of the original tryptic digest. In the present paper evidence is adduced to show that the remaining organic phosphorus is present in similar if not identical form, and an account is given of the isolation and identification of the individual units of phosphopeptone, together with a suggested structure.

Isolation of the amino-acids in phosphopeptone.

20 g. of phosphopeptone were hydrolysed by boiling with 20 % hydrochloric acid for 36 hours. The resulting solution was concentrated and saturated with hydrogen chloride. After 4 days in the ice-box and a second saturation no material had crystallised except a small quantity of ammonium chloride. Hydrochloric acid was removed from the liquid by repeated concentration *in vacuo*; the resulting syrup was then diluted to about 200 cc. and the amino-acids were converted into their calcium salts by the method of Foreman [1914]. The phosphoric acid was at the same time converted into calcium phosphate and removed with the excess of lime by filtration.

After concentration *in vacuo*, the salts of the dibasic acids were precipitated by 97 % alcohol, about 2 l. being required. The sticky precipitate was dissolved in water and calcium removed quantitatively with oxalic acid. The solution was then concentrated and saturated with hydrochloric acid gas.

No glutamic acid hydrochloride could be separated even after long standing. Hydrochloric acid was therefore removed by repeated evaporation *in vacuo*, and the amino-acids converted into their copper salts by boiling with fresh copper hydroxide. The dark blue solution was concentrated and allowed to stand at 0° but nothing crystallised out. Aspartic acid was therefore absent.

Copper was removed from the solution by hydrogen sulphide and traces of chloride by acid silver nitrate, sodium hydroxide was then added to neutrality and the silver salt of hydroxyglutamic acid precipitated as described by Dakin [1918]. Thus obtained it was nearly pure, and, after one reprecipitation, the silver salt was analysed.

Analysis. $C_5H_7O_5NAg_2$.

	C	H	N	Ag
Found	15·42	1·75	3·5	57·5 %
Calculated	15·9	1·85	3·7	57·3 %

Yield: just over two molecules per molecule of phosphopeptone. For further identification the copper salt was prepared by boiling a solution of the acid, prepared from the silver salt, with excess of freshly precipitated copper hydroxide, filtering, concentrating, and precipitating the copper salt by alcohol.

Analysis. $C_5H_7O_5NCu$.

	C	H	N	Cu
Found	25·1	3·50	5·8	27·4 %
Calculated	26·7	3·12	6·2	28·3 %

As the copper salt is not very suitable for analysis and as difficulty was experienced in getting the silver salt quite free from silver oxide, the free acid was also prepared by decomposing 1·5 g. of the silver salt with hydrogen sulphide and concentrating the resulting solution over sulphuric acid *in vacuo* at room temperature. It was obtained (0·7 g.) in microcrystalline form and corresponded with Dakin's [1918] description, giving all the colour tests described by him. When heated it softened indefinitely at about 100°. For analysis a sample was dried over phosphorus pentoxide at 35–40°.

Analysis. Ash = 1·2 %.

	C	H	N
Found (ash-free)	36·5	5·60	8·60 %
Calc. for $C_5H_9O_5N$	36·8	5·52	8·59 %

The only dibasic acid present in the peptone is hydroxyglutamic acid. Its nitrogen corresponds to between two and three atoms out of the total of nine in the molecule.

The alcoholic solution of the calcium salts of the monobasic acids was concentrated *in vacuo* and calcium was removed by oxalic acid. The resulting solution gave a precipitate with neutral lead acetate, which proved to be only lead oxalate, due to a slight excess of oxalic acid in the solution. No lead salts were precipitated from the filtered solution by the addition of ammonia. Lead was removed from the filtrate as sulphide. The resulting liquid was concentrated to a small bulk *in vacuo* at 37° and the concentration continued over sulphuric acid *in vacuo* at room temperature. A syrup was obtained which gradually deposited a fairly large quantity of crystalline material.

Under the microscope two types of crystal were visible; in the greater proportion, very small platelets or prismatic needles, frequently grouped

together in dense aggregates, and among these large, rather irregular plates similar in appearance to crystals of serine.

A method of separation based upon chemical properties was sought without success. An attempt was therefore made to achieve separation by fractional crystallisation of the remainder of the material.

Differences in solubility were not great, but the more abundant substance was eventually obtained in a fairly pure state. It was very hygroscopic and had to be dried to constant weight over phosphorus pentoxide *in vacuo* at 50° before analysis. The figures obtained agree most nearly with the formula $C_4H_9O_3N$ which is that of hydroxyaminobutyric acid, a homologue of serine.

Analysis (micro.).

	C	H	N
Found	41·18	8·83	11·67 %
Calc. for $C_4H_9O_3N$	40·34	7·56	11·77 %

The copper salt was prepared by boiling a solution of the acid with freshly precipitated copper hydroxide. On concentration the liquid deposited small prismatic crystals having an intense deep blue colour. The copper salt of hydroxyaminobutyric acid was found by Schryver and Buston [1926] to contain two molecules of water of crystallisation.

Analysis. $(C_4H_8O_3N)_2Cu.2H_2O$.

	N	Cu
Found	8·60	19·73 %
Calculated	8·34	18·93 %

The dibenzoyl derivative was prepared by adding 3 cc. benzoyl chloride and 7 cc. 5*N* sodium hydroxide to a solution of 0·5 g. of the acid in 10 cc. water. After acidification, the precipitated product was extracted repeatedly with boiling light petroleum to remove benzoic acid and the insoluble residue was crystallised from a little hot benzene. It crystallised in fine needles having M.P. 109°. Schryver gives the M.P. as 112°.

The second fraction was small in amount and was consequently more difficult to obtain in a pure state. It crystallised in flat plates, was hygroscopic and was analysed after desiccation over phosphorus pentoxide.

Analysis. Nitrogen 12·60 %; Ash 2·83 %; Nitrogen (ash-free) 12·97 %.

The nitrogen content of serine is 13·33 %.

The substance melted with decomposition at 223°. The M.P. of *l*-serine is 224°. It also gave a benzoyl derivative which crystallised in fine needles, M.P. 121°. Dibenzoylserine melts at 124°.

Lack of material prevented further methods of identification, but from the above data it appears probable that the substance under investigation was an impure specimen of serine.

No other amino-acids were found.

The difficulty of separating the hydroxyamino-acids from one another and from other amino-acids is well known. In the above case the yield of hydroxyglutamic acid corresponded to between two and three molecules per molecule

of phosphopeptone, but the yields of hydroxyaminobutyric acid and of serine were considerably smaller.

The possibility exists that phosphopeptone may contain some hitherto unknown amino-acid, nevertheless, assuming that only the three which have been isolated are present, namely, hydroxyglutamic acid, hydroxyaminobutyric acid and serine, in the relative proportions depicted below, a formula can be arrived at for the peptone which agrees with that indicated by elementary analysis.

The molecule of phosphopeptone would thus appear to be made up of the following:

Hydroxyglutamic acid	3 molecules
Hydroxyaminobutyric acid	4 ,,
Serine	2 ,,
Phosphoric acid	3 ,,

Hydroxyaminobutyric acid has so far been isolated from only one protein, namely, from oat glutelin by Schryver and Buston [1926]. The above scheme would require its presence in caseinogen to the extent of 1·8 % or, if the whole of the phosphorus is similar to phosphopeptone, of about 3·6 %. Similarly the serine would amount to 0·8 % or 1·6 % of the protein. The best yield of serine from caseinogen so far recorded is 0·5 % but the amount actually present is undoubtedly greater [cf. Emil Fischer, 1903].

Constitution of phosphopeptone.

In endeavouring to form a picture of the way in which the units of this peptone are united certain requirements, determined by experiment, must be fulfilled, namely:

(i) The molecular formula shall be $C_{37}H_{62}O_{33}N_9P_3$.

(ii) The substance must behave as a nine-basic acid.

(iii) One-ninth of the total nitrogen shall be present in the form of amino-nitrogen, the remaining linkages must be peptide linkages.

(iv) An explanation must be offered of the fact that two-thirds only of the total phosphorus is removed by bone phosphatase in the form of phosphoric acid, the remaining one-third being however almost certainly also in ester linkage as it is removed by the phosphoric esterase of kidney extracts [see Rimington and Kay, 1926].

Whilst it must be emphasised that as yet there is no possible means of deciding to *which* of the constituent amino-acids the phosphorus atoms are attached, nor in what sequence they occur, a structural formula can be constructed which satisfies *all* of the above conditions. This formula is depicted in Fig. 1 but it must be remembered that the positions of the amino-acids and phosphorus atoms are chosen arbitrarily.

It will be seen that two of the molecules of phosphoric acid are visualised as being united through one hydroxyl group only to the amino-acid chain, whilst the third forms a di-ester linkage. This affords a ready explanation of

the difference between the action of bone and of kidney phosphatase, a fact which was difficult to interpret upon any other basis[1].

Subsequent work may demonstrate the exact positions of the phosphorus groups, but in the meantime it was thought advisable to give the above formula for the sake of descriptive clearness.

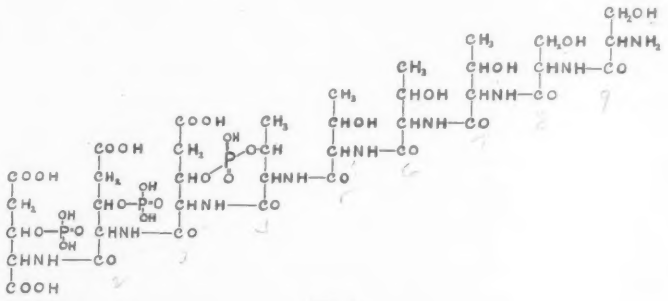


Fig. 1.

(3 molecules of hydroxyglutamic acid) (4 molecules of hydroxyaminobutyric acid) (2 molecules of serine

The relation of phosphopeptone to the remainder of the caseinogen phosphorus.

As has been pointed out, the yield of pure phosphopeptone prepared from tryptic digests of caseinogen represents 50 % only of the original organic phosphorus. When however the action was investigated of bone and kidney phosphatase upon phosphopeptone and upon neutralised tryptic digests respectively a very striking similarity was observed. In both cases only two-thirds of the total organic phosphorus was hydrolysed by bone phosphatase preparations, the remainder being liberated by the kidney enzyme. These results are shown in the two figures previously published [Rimington and Kay, 1926, p. 783, Fig. 4; Rimington, 1927, 1, p. 278, Fig. 2].

It appears probable therefore that if other substances analogous to phosphopeptone exist, the form of the phosphorus linkages in them must be similar to those in phosphopeptone itself. The peptide linkages of phosphopeptone appear to be fairly resistant to tryptic hydrolysis; on the other hand, the peptone is split off from the rest of the caseinogen molecule with such rapidity that the linkages between it and the parent grouping must be of a labile nature and readily attacked by trypsin.

To the above considerations is due, no doubt, the fact that the material isolated from different preparations has, when pure, always the same composition.

[1] Martland and Robison [1927] have recently shown that bone phosphatase has very little action upon the diethyl ester of phosphoric acid while the monoethyl ester is readily hydrolysed.

Discussion.

The association of phosphoric acid in the caseinogen molecule with a complex of hydroxyamino-acids raises several interesting questions. No evidence is as yet available concerning either the specific metabolic importance of the hydroxyamino-acids as a class, or the nutritional significance, if any, of phosphoric acid united with them in such a compound as the phosphopeptone just described. The behaviour of this peptone as a whole, its ready diffusibility and resistance to trypsin in no way exclude the possibility of its absorption from the gut into the blood-stream unchanged.

Its ready hydrolysis by the enzymes of ossifying cartilage also makes the suggestion not improbable that the phosphorus of caseinogen may by this means contribute in part to the inorganic phosphate laid down in bone formation. Experiments bearing upon this aspect of the problem are now in progress.

Caseinogen has frequently been regarded as a conjugated protein, the phosphorus it contained having been assumed to be present in some prosthetic group. The association seen in phosphopeptone cannot be regarded as of this kind, *i.e.* one in which constituents of a non-protein nature are characteristically present.

During the course of proteolysis, fragments, some large and some small, are formed by cleavage of the protein under the action of the enzyme. Some of these fragments show comparative resistance towards further hydrolysis, and phosphopeptone, however built into the general architecture of the caseinogen molecule, must be regarded as of this type.

Many proteins other than caseinogen contain phosphorus, although usually to a much smaller extent. It is, however, impossible to state whether or not in these cases also the association is one of phosphoric acid with hydroxyamino-acids. If such should prove to be the case, it might be permissible to regard the combination as a new unit constituent of proteins in general, such as, for example, phosphorylglutamic acid, which would be related to hydroxyglutamic acid in much the same way that cysteine is to serine.

Summary.

1. From phosphopeptone after hydrolysis have been isolated hydroxyglutamic acid, hydroxyaminobutyric acid and serine.

2. From the above data a hypothetical structural formula has been constructed that is in agreement with all other known chemical properties of the peptone.

In conclusion I wish to thank Sir F. G. Hopkins for the interest he has taken in this work and also Dr H. D. Kay for encouragement and advice.

Note in conjunction with H. D. Kay.

S. Posternak recently called our attention [Posternak, 1927, 1] to work of his own on a patented process for "preparing the phosphorus nucleus of caseinogen" which had appeared (unaccompanied by the author's name) in a Swiss Patent Specification in 1923. Although we had examined the literature, this specification had unfortunately escaped our notice. Lately [1927, 2] he has claimed to have isolated from caseinogen a pure phosphopolypeptide which contains the following amino-acids: glutamic acid, 3 mols.; aspartic acid, 0·6 mols.; *iso*leucine, 3 mols.; serine, 3 mols.; phosphoric acid, 4 mols. From these findings he comes to the rather surprising conclusion that the phosphorus nucleus of caseinogen is composed of four serine-phosphoric acids.

Knowing some of the difficulties and pitfalls attending this work of purification and analysis, we are of opinion that it is only in the light of considerably more experimental and analytical detail than is presented by Posternak that his conclusions regarding the chemical composition of the "phosphorus nucleus of caseinogen" can be assessed.

If we take his statements at their face value we have no alternative but to conclude that the phosphopeptone first obtained by us in a crude form in 1924 (described at a meeting of the Biochemical Society on Feb. 9th, 1925 [Rimington and Kay, 1925]), and which has now been purified and analysed by one of us, is entirely different from the "pure" substance described by Posternak.

REFERENCES.

Dakin (1918). *Biochem. J.* **12**, 290.
Fischer (1903). *Z. physiol. Chem.* **39**, 155.
Foreman (1914). *Biochem. J.* **8**, 463.
Martland and Robison (1927). *Biochem. J.* **21**, 665.
Posternak (1927, 1). *Biochem. J.* **21**, 289.
—— (1927, 2). *Compt. Rend. Acad. Sci.* **184**, 306.
Rimington (1927, 1). *Biochem. J.* **21**, 272.
—— (1927, 2). *Biochem. J.* **21**, 1179.
Rimington and Kay (1925). *Chem. Ind.* **44**, 256.
—— —— (1926). *Biochem. J.* **20**, 777.
Schryver and Buston (1926). *Proc. Roy. Soc. Lond.* B, **99**, 476.

CLIX. OBSERVATIONS ON SOME PROCESSES OF OXIDATION IN BLOOD-SERUM.

By THOMAS RICHARD PARSONS and WINIFRED PARSONS.

From the McGill University Clinic, Montreal, Canada.

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The usual method for the estimation of the amount of oxygen combined as oxyhaemoglobin in a sample of blood consists in treating the fluid with potassium ferricyanide in faintly alkaline solution, when a complicated reaction occurs in which methaemoglobin is formed and an amount of oxygen equal to that originally present as oxyhaemoglobin is set free for measurement. But that the change which takes place when ferricyanide is added to so complicated a mixture as blood is completely expressed by this simple statement has long been questioned. Among the more recent work bearing on this point we may mention Conant and Fieser's [1924] demonstration of the dependance of the completeness of the reaction between potassium ferricyanide and oxyhaemoglobin on the degree of alkalinity of the medium in which that reaction takes place, and also the results of Smith, Dawson and Cohen [1920] which prove that in the van Slyke method of applying the ferricyanide reaction—a method in which the liberated oxygen is extracted at once from the reaction mixture by exposure to a vacuum—an appreciably greater amount of oxygen is liberated from a given quantity of blood than is obtained by the procedure of Henderson and Smith, in which the blood and ferricyanide are merely shaken together in a known volume of air and the amount of oxygen liberated is measured by the resulting increase in the percentage of oxygen in the gas phase. But apart from these and earlier investigations of the completeness of the reaction between ferricyanide and oxyhaemoglobin the observation has also been made that, when the oxygen has been set free in this way, if the shaking and exposure be continued, the liberated gas is slowly re-absorbed by the reaction mixture. This phenomenon is most marked with blood from anaemic patients, and with such blood it may proceed at such a rate as to render impossible any accurate estimation of the volume of oxygen originally liberated. The effect is so definite and of such magnitude that it seemed desirable to make an attempt to obtain some more precise knowledge as to the factors and substances involved when a mixture of blood and potassium ferricyanide absorbs oxygen under these conditions, and the results recorded in the present paper represent the information that we have collected.

For these experiments we used a compact form of Haldane's [1920, 1922] new blood-gas apparatus that is represented diagrammatically in Fig. 1. The main features are that the long rubber tubes of the original form of the apparatus are eliminated, and that the whole apparatus can be immersed in a thermostat bath through the glass sides of which the readings are taken. The zero line of the U-tube manometer is drawn on a small mirror which is arranged so that it is adjustable in a vertical direction in order to allow for slight variations in the level at which the manometric liquid (xylene) stands. The gas-measuring burette has a total volume of 0·2 cc. and is graduated in 0·002 cc. so that using mercury as confining fluid readings of gas volume correct to $\pm$ 0·0002 cc. can be obtained. This degree of accuracy is, however, far greater than that required for the purposes of this particular piece of work.

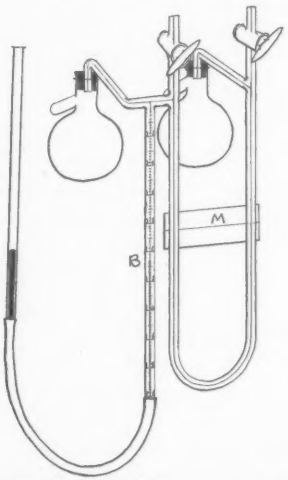

Fig. 1. Compact form of Haldane blood-gas apparatus. *B* is the gas-measuring burette containing mercury as confining fluid, and *M* the adjustable mirror carrying the zero line for the manometer U-tube.

The apparatus is most conveniently mounted on a plate of aluminium about $\frac{1}{8}$ in. thick with a suitable slot for receiving the mirror and its clamping screw.

Our ordinary procedure has been to place 1 cc. of the blood or other material under investigation together with 2 cc. of dilute ammonia solution (4 cc. of a solution of d. = 0·880 diluted to 1 l.) or other alkaline solution in the reaction-bottle and to place a similar mixture containing 1 cc. of distilled water in place of the blood or serum on the control side. We used in the side-tube of each bottle 0·5 cc. of a saturated solution of potassium ferricyanide, and from the final colour of our reaction mixtures it was evident that this always constituted a great excess. The bottles having been fitted to the rest of the apparatus, the whole was lowered into the thermostat and when temperature equilibrium had been attained the taps were closed and the

ferricyanide tilted into the experimental material. The apparatus was shaken by hand for 2 minutes and then replaced in the bath for a further 3 minutes. By adjustment of the mercury reservoir tube the level of the xylene was brought to the zero line on the mirror and the reading of the gas volume then noted. This gave a starting-point for the observations of the subsequent absorption of oxygen, which was followed usually for about an hour by shaking the apparatus for 2 minutes and allowing it to rest in the bath for the following 3 minutes before each reading. By this procedure we, of course, systematically neglected the amount of oxygen absorption occurring during the first 5 minutes of each experiment; we did this in order to avoid occasional irregularities due to the mixing of the materials or to evolution of gas from small quantities of haemoglobin present, and all the curves we give are plotted from results consistently obtained in this way. But we have convinced ourselves that no appreciable difference is produced by the inclusion of these initial 5-minute intervals in the diagrams and that our conclusions are therefore in no way

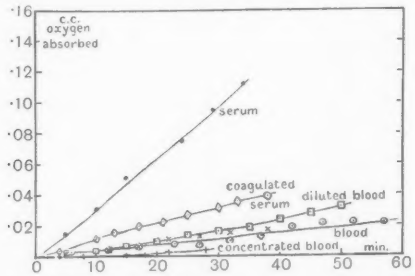

Fig. 2. Effect of variation of the concentration of corpuscles on the rate of absorption of oxygen by blood in the presence of ferricyanide. The points denoted by crosses were obtained in the presence of NaF. The oxidation curve for coagulated serum is also given.

affected either qualitatively or quantitatively thereby. All our experiments were carried out at room temperature, which rarely varied outside the limits of 15–18°, and all the gas volumes recorded in our diagrams have been reduced to N.T.P.

With this technique it was easy to show, in the first place, that the absorption of oxygen by a mixture of defibrinated blood and ferricyanide is due entirely to the serum and that the corpuscles play no part in it. For example in Fig. 2 we record the results of observations made on the blood of a male patient, S., who possessed a haemoglobin content of 60 % of normal and a red cell count of $3{\cdot}44 \times 10^6$ per mm.^{3}. It will be seen that doubling the concentration of the corpuscles by centrifuging almost completely abolishes the absorption of oxygen, while dilution of the blood with an equal volume of its own serum very considerably increases it. The rate at which the pure serum itself takes up oxygen in the presence of ferricyanide is very considerable, amounting to about 0·1 cc. per cc. of fluid in 30 mins. It is evident, then, that we are not here dealing with an absorption of oxygen by immature

red corpuscles such as might have been expected to occur in the blood of an anaemic patient, and that the oxidation is so much more marked in this than in normal blood simply because the anaemic blood contains the larger proportion of serum. As a matter of fact the rate of this oxygen absorption in the whole untreated blood even of this patient is such as might easily be overlooked in an ordinary oxygen determination, and in the blood of a normal person would be even less. With the serum from this same blood we convinced ourselves that absorption of oxygen does not occur in the absence of ferricyanide, obtaining a volume difference of only 0·0005 cc. during 25 minutes' shaking of the serum alone in air. We also showed that this oxidation is not dependent on a process of glycolysis inasmuch as the rate of absorption of oxygen by 1 cc. of this blood was totally unaffected by the addition of 0·2 cc. of 0·5 % sodium fluoride solution according to Evans' recommendation [1922] (see the points represented as crosses in Fig. 2). We further obtained evidence that we were not dealing with an enzyme reaction of any kind by observing the rate of absorption of oxygen by 1 cc. of serum that had been heated by holding the bottle in boiling water for 5 mins. before the experiment. Certainly in this case the rate of oxidation was much reduced (Fig. 2), but this is readily to be explained by the slow rate of diffusion and the difficulty of adequate shaking caused by the presence of large masses of coagulum.

In considering the various constituents of serum that might be responsible for the formation of autoxidisable substances under the influence of potassium ferricyanide we first of all suspected that the effect might be due to sugar. But we found that, under the conditions of our experiment—that is, in faintly alkaline solution—1 cc. of 0·5 % glucose solution absorbed no appreciable amount of oxygen when shaken with air in the presence of ferricyanide, the actual change in volume observed at the end of an experiment lasting 41 mins. amounting to only 0·0008 cc.

We were left, therefore, with the alternative possibilities that the autoxidisable constituents arise either from the proteins of the serum, or from the lipins, or possibly from a colloidal complex of the two. We therefore attempted to fractionate the serum in various ways and to study the oxidisability of the separate fractions. We tried, for example, to extract the lipins by shaking with ether, and found that the residual aqueous fluid showed a much slower rate of oxygen absorption than the original serum, and that this reduction of oxidisability was the more marked the more efficient the ether extraction. When the ether extract was evaporated to dryness in a dish floating on warm water a residue was obtained that turned brownish during the last stages of the evaporation. When this residue was re-emulsified in some of the extracted serum no appreciable increase in the rate of oxidation was produced, undoubtedly because of oxidation of the lipins during the evaporation. When we repeated the experiment in such a way that the ether extraction and also the evaporation of the extract were carried out in an atmosphere of nitrogen, we found that when the lipin residue obtained was re-emulsified in the extracted

serum the rate of oxygen absorption was markedly increased. The result of this experiment is recorded in Fig. 3 in which it is to be noted that the curve labelled "extracted serum + lipins" was obtained with 1 cc. of extracted serum to which the lipins obtained from 1·5 cc. of the original serum had been added, so that in this experiment the effect of the addition of the lipins is exaggerated. In passing we would call attention to the particularly rapid rate of absorption of oxygen shown in this experiment by the mixture of untreated serum and ferricyanide, 0·24 cc. oxygen being taken up by 1 cc. serum in less than 50 mins. This is associated with the circumstance that the blood used in this experiment was obtained from a patient, F., exhibiting so marked a degree of lipaemia that the serum was opaque and milky with the excessive amount of fat it contained. We have confirmed these effects of the ether extraction and re-addition of lipins using serum containing only a normal content of these substances; we have found further that extraction with light

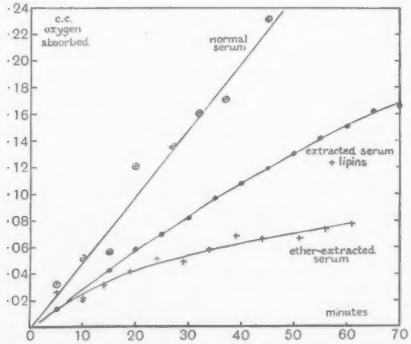

Fig. 3. Effect of ether extraction and re-addition of the lipins obtained by evaporation of the extract out of contact with air.

petroleum also reduces to a similar extent the rate at which the serum will absorb oxygen in the presence of ferricyanide. The fact that extraction with these lipin solvents reduces the oxidisability of the serum does not necessarily indicate that the lipins themselves are concerned in the effect, for, these solvents produce a certain amount of precipitation of the serum-proteins. This effect is not very marked in the case of ether, but with light petroleum emulsion formation is very troublesome, and vigorous shaking of the mixture of serum and petroleum results in an emulsion of the consistence of vaseline, which separates only imperfectly into petroleum and aqueous layers in the centrifuge, a compact mass of material giving protein reactions separating between the two main liquids. The reduction of the oxidisability of the serum described in these experiments might therefore well be due to an effect of the extracting fluid on the proteins, but the fact that the re-addition of the extracted lipins does in some measure increase the rate of oxidation of the extracted serum can be interpreted only as indicating a direct participation

of the lipins in the process of oxidation here under study. By re-addition of the extracted lipins, however, we have never succeeded in restoring the rate of oxidation to that shown by the original unextracted serum, but whether this incompleteness of the restoration of the oxidisability is due merely to incompleteness of mechanical dispersion we are at present not able to say.

In view of the evidence that exists in favour of the view that the lipins of the serum are present in the form of colloidal complexes with the proteins, we carried out also an experiment on the separation of the proteins and lipins of the serum by the method described by Hardy and Gardiner [1910]. Serum was run into some six times its volume of alcohol previously cooled to $-8°$; the precipitated proteins were filtered off at a temperature not rising above $0°$, then freed from alcohol by thorough washing with ether and finally dried in the vacuum desiccator. One half of this material was dissolved in a volume of water equal to half that of the serum originally taken, and the oxidation of this solution in the presence of ferricyanide was observed. The lipins were recovered from the combined filtrates by evaporation *in vacuo*. The residue was emulsified by shaking in a volume of distilled water

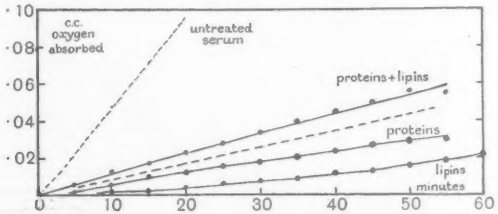

Fig. 4. Rate of oxidation in the presence of ferricyanide of the proteins and lipins separated from serum by ice-cold alcohol. The unlabelled dotted line represents the arithmetical sum of the curves for lipins and proteins respectively.

equal to that of the original serum, the process being conducted in an atmosphere of hydrogen. One half of the resulting emulsion was used for an oxidation experiment, while in the other the remaining half of the protein precipitate was dissolved by shaking in hydrogen to yield a "resynthesised" serum whose oxidisability was also studied. The results of this experiment are shown in Fig. 4 from which it will be seen that both the protein and the lipin fractions take up oxygen slowly in the presence of ferricyanide, and that the mixture of the two, in the proportions and concentrations in which they originally existed in the serum, absorbs oxygen at a rate hardly greater than that to be expected from the arithmetical summation of the effects due to the two constituents separately. From this it would seem that both proteins and lipins are concerned in the process of oxidation and that neither exerts an appreciable accelerating influence on the oxidation of the other. If a lipin-protein complex is re-formed under the conditions of our experiments it does not show any appreciably greater susceptibility to oxidation than is possessed by its separate lipin and protein constituents. It will be noticed, however

how far we were in this experiment from achieving a re-synthesis of the original serum by a re-union of its separated constituents: it is evident that the process of separation had produced deep-seated and irreversible changes of the colloidal structure of the serum and consequent reduction in the rate of oxidation—an effect apparently analogous to the diminution of the rate of oxygen absorption observed by Warburg [1914] to follow successive destruction of structure in nucleated red blood cells.

Our next observations are concerned with the fractionation of the serum by means of ammonium sulphate. A first experiment showed that when serum is saturated with this salt the oxidisable substances are completely precipitated—the filtrate absorbing no oxygen at all when treated with ferricyanide in the presence of ammonia according to our usual procedure.

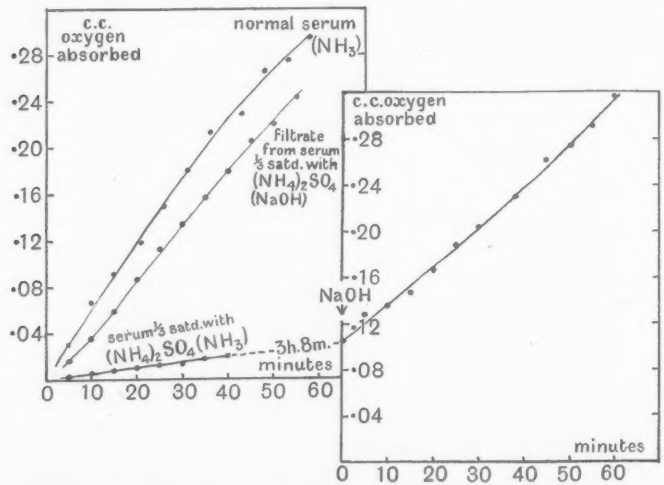

Fig. 5. Effect of one-third saturation with ammonium sulphate and subsequent addition of NaOH. The alkali present during the oxygen absorption is mentioned in brackets at the end of the description of each curve.

But the precipitated proteins were also practically devoid of oxidisable properties when treated in this way. This result suggested that possibly the large amount of ammonium sulphate present in these experiments had depressed the ionisation of the ammonia used as alkali to such an extent that the final mixture was far less alkaline than in our previous experiments, and that therefore the oxidising potential of the ferricyanide was so much reduced that it was incapable of giving rise to the autoxidisable substances whose behaviour we are here studying. It is possible also that the rate of absorption of oxygen by these autoxidisable substances themselves might have been reduced by a reduction in the alkalinity of the medium. We therefore proceeded to the experiment whose results are recorded in Fig. 5. In this case

we compared the rate of absorption of oxygen by untreated serum in the presence of ammonia with that shown by serum mixed with one-third its volume of saturated ammonium sulphate solution. In this mixture the rate of oxygen absorption was very slow until 1 cc. of approx. *N* NaOH solution was added at the point indicated. The result of the increased alkalinity was shown in an immediate increase in the rate of oxidation to a value not far different from that of the untreated serum. Evidently, then, the depressant effect of the ammonium sulphate is to be ascribed to the reduction it produces of the alkalinity of the reacting mixture. In Fig. 5 we include also the results of an experiment on the *filtrate* from serum one-third saturated with ammonium sulphate, and examined in the presence of 1 cc. *N* NaOH instead of ammonia. It is evident that one-third saturation with ammonium sulphate does not precipitate any appreciable amount of the substance or substances responsible for the oxidations we are studying—a result confirmed by experiments described below.

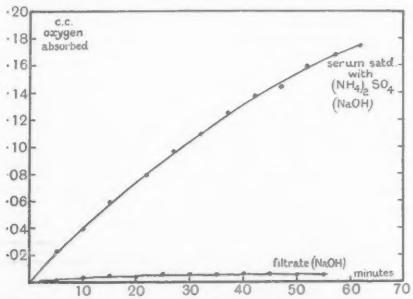

Fig. 6. Precipitation of the oxidisable materials by full saturation of the serum with ammonium sulphate.

On repeating now the experiment with full saturation with ammonium sulphate and using 1 cc. *N* NaOH as alkali it was easy to show that this procedure precipitated completely the oxidisable substances, for the filtrate from serum saturated with ammonium sulphate showed no oxygen uptake even in strongly alkaline solution, while the unfiltered mixture of serum and ammonium sulphate under the same conditions absorbed oxygen at a rate commensurate with that observed with the untreated serum (Fig. 6). The oxidisable material was therefore contained in the precipitate. A further series of observations illustrating the successive precipitation of the active material by ammonium sulphate is recorded in Fig. 7, from which it will be seen that, as mentioned above, one-third saturation with the salt removes only a small portion of the oxidisable substances while half saturation brings down a much larger fraction. In all these experiments we used such an amount of each of the various filtrates as corresponded to 1 cc. of the original serum, and added ammonium sulphate solution to the reaction mixtures in

such amounts that the concentration of this substance, together with the amount of NaOH and the total volume of the liquid under test, was the same for all experiments of any one series, so that they are strictly comparable *inter se*. As we were able to confirm the results of Fig. 7 on another sample of serum we may state definitely that the oxidisable substances are precipitated completely by full saturation with ammonium sulphate, partially by half saturation and hardly at all by one-third saturation with ammonium sulphate.

Seeing that from the results of the ether extraction experiments we concluded that the lipins of the serum are to a large extent concerned in the oxidations we are considering, we were naturally led to compare the behaviour of lecithin emulsions towards ammonium sulphate with that of the serum itself. It is easy to show that a 1 % emulsion in distilled water of Merck's lecithin (prepared from eggs) is rapidly and completely precipitated by saturation with ammonium sulphate, less rapidly but still as completely by half saturation and that even one-third saturation with the salt will throw out the

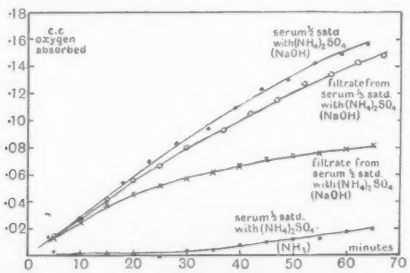

Fig. 7. Progressive precipitation of the oxidisable materials by increasing concentrations of ammonium sulphate.

whole of the lipin in the space of 2 to 3 hours. As the liquids from which the results of Fig. 7 were obtained were allowed to stand overnight before filtering, it might have been expected that the precipitation of the lipins would have been complete even in the case of the serum only one-third saturated with ammonium sulphate. But it is also easy to show that the presence of proteins very much modifies the conditions of precipitation of lecithin by salts. For example, if a mixture of 1 cc. of a 1 % emulsion of lecithin lightly stained with Sudan III and 1 cc. of 1 % gelatin solution be added to 10 cc. $N/100$ NaOH and the solution be then one-third saturated with ammonium sulphate by the addition of 6 cc. of the saturated solution, it is evident from the red colour remaining in the liquid that the bulky precipitate that is produced does not contain the whole of the lipin, although, in the absence of the protein, lecithin itself is completely precipitated under these conditions. In this alkaline solution the protein exerts a protective action on the lecithin. If we can assume that similar relationships obtain between the proteins and the lipins of the serum we can at once account for our observations.

These results have a bearing on the general question of the relationship of lipins to proteins in serum. As Chick demonstrated [1914], the euglobulin of serum is a complex of pseudoglobulin and phospholipin and is precipitated by one-third saturation of the serum with ammonium sulphate. That this procedure does not render the serum lipin-free is evidently due to the fact that the serum-proteins exert a protective action on the lipins similar to that which we have observed to be exerted on lecithin by gelatin: we can thus account for Chick's observation that after precipitation of the euglobulin from serum by one-third saturation with ammonium sulphate there remains sufficient lipin in the filtrate to combine slowly with pseudoglobulin to form a further quantity of euglobulin.

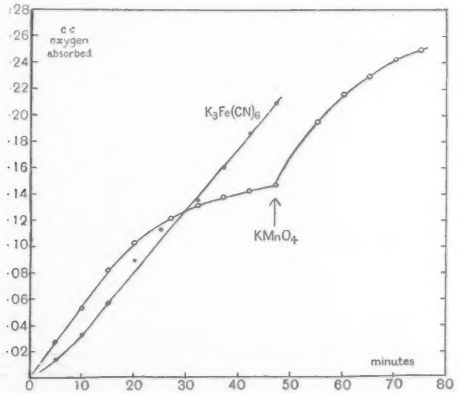

Fig. 8. Effect of substitution of potassium permanganate for ferricyanide: at the arrow a second portion of permanganate was added to the serum.

We should mention lastly that we made a few experiments to determine the effect of substituting other oxidising agents for the ferricyanide used in all the experiments so far described. As is seen in Fig. 8, for example, potassium permanganate in dilute ammonia solution acts even more powerfully in promoting the oxidation than ferricyanide, but the effect gradually passes off owing to the fairly rapid reduction of the permanganate by the proteins and other oxidisable constituents of the serum. On adding a further quantity of permanganate the rate of oxygen uptake is once more increased. So far as we are aware there are no experiments on the oxidation of lecithin in the presence of potassium permanganate with which we can compare this result obtained with serum. With potassium dichromate, which from the observations of Thunberg [1916] is known to accelerate the oxidation of ordinary lecithin, we obtained no acceleration of the oxidation of serum comparable with that produced by ferricyanide and permanganate, and this no matter whether we worked in an alkaline medium (1 cc. *N* NaOH + 1 cc. saturated ammonium sulphate being added to 1 cc. of the serum) or in an acid medium,

in which case 2 cc. approx. 0·1 *N* acetic acid were added to 1 cc. of serum (Fig. 9). Using ferric chloride also we found no appreciable absorption of oxygen to take place in the acid medium, but when the alkaline mixture was used the rate of oxygen uptake after a short initial delay due probably to the difficulty of efficient shaking of the mixture before the bulky precipitate of ferric hydroxide had shrunken together, was no less rapid than in the presence of ferricyanide (Fig. 9). This result again is just the opposite of that obtained by Warburg and Meyerhof [1913] with emulsions of ordinary lecithin, for they found that iron was a more efficient catalyst of the oxidation in acid solution than in neutral or alkaline.

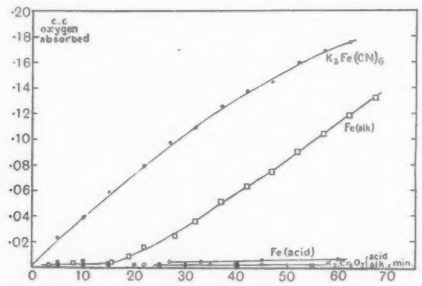

Fig. 9. Substitution of ferric salts and potassium dichromate for ferricyanide in the oxidation of serum.

It is natural to compare the results of this investigation with those obtained by Harris [1926] in his work on the oxidation of blood-plasma exposed to light. He found that when exposed to light 1 cc. plasma absorbs oxygen at the rate of about 0·024 cc. per hour, and ascribed this effect to the tyrosine and tryptophan present in the molecules of the plasma-proteins. But as this rate of oxidation is of the order of one-tenth of that which we have observed in the serum in the presence of ferricyanide, and, further, as Harris found that the addition of ferricyanide produces a marked degree of protection of the plasma against oxidation under the conditions of his experiments, it is evident that the processes of oxidation that occur when plasma is exposed to light can account for only a small fraction of the total oxidation that we have observed in the serum in the presence of ferricyanide.

In conclusion we would add that we do not claim any measure of completeness for the data and explanations here presented, but we have thought it worth while to publish in this form the results we have obtained so far on account not only of their practical bearing on the technique of blood-gas analysis, but also of their relation to the general study of biological oxidation systems.

SUMMARY.

In the presence of certain oxidising agents such as potassium ferricyanide and potassium permanganate human blood-serum absorbs oxygen at the rate of about 0·25 cc. per cc. per hour when shaken with air at room temperature. The same effect is observed in the presence of ferric salts in alkaline solution, but not with ferric salts in acid solution nor with dichromate at any reaction. Extraction with ether reduces the rate at which the serum will absorb oxygen in this way, but this can be restored in some measure by the re-addition of the extracted material provided that this is recovered by evaporation of the ether extract in an oxygen-free atmosphere. This suggests that the substances primarily responsible for the effect are lipins and this explanation fits in with the further observations that we have made on serum fractionated by means of ammonium sulphate, when the conditions determining the precipitation of lipins by this salt in the presence of proteins are taken into account.

REFERENCES.

Chick (1914). *Biochem. J.* **8**, 404.
Conant and Fieser (1924). *J. Biol. Chem.* **62**, 595.
Evans (1922). *J. Physiol.* **56**, 146.
Haldane (1920). *J. Path. Bact.* **23**, 443.
—— (1922). Respiration. (Yale University Press).
Hardy and Gardiner (1910). *J. Physiol.* **40**; *Proc. Physiol. Soc.* lxviii.
Harris (1926). *Biochem. J.* **20**, 271.
Smith, Dawson and Cohen (1920). *Proc. Soc. Exp. Biol. Med.* **17**, 211.
Thunberg (1916). *Skand. Arch. Physiol.* **33**, 228.
Warburg (1914). *Ergebn. Physiol.* **14**, 320.
Warburg and Meyerhof (1913). *Z. physiol. Chem.* **85**, 412.

CLX. THE SPECIFIC ACTION OF PLANT ENZYMES.

III. THE SPECIFIC CONDITIONS OF ACTION OF LEAF SALICINASES.

By A. V. BLAGOVESCHENSKI and N. I. SOSSIEDOV.

From the Laboratory of Plant Physiology, State University, Tashkend.

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We have investigated the action of air-dried powdered leaves of some species of *Salix* and *Populus* on salicin solutions at various p_H values. The leaves of all the species of *Salix* and *Populus* (except *S. hastata*) were collected at Tashkend, dried rapidly at 25–30° and ground in a mortar to the state of fine powder. 0·2 g. of this powder was mixed with 10 cc. of McIlvain's [1921] buffer solution (citric acid and Na_2HPO_4) and with 5 cc. of 3 % salicin (Kahlbaum) solution. To all experimental flasks toluene was added and they were kept at 30–33°. For control the same conditions, but without salicin, were maintained. The determinations of glucose liberated were made by the Benedict-Lewis picric acid method, as we described in our previous paper [1925]. The p_H values were determined colorimetrically with Clark and Lubs' indicators. The full details are given for Exp. 1, but in all the other experiments only the difference between the amounts of reducing sugar formed in the presence and absence of salicin are quoted under the heading "Change in reducing sugar."

Exp. 1. The leaves of *P. Bachofenii* Wierzb., collected 13. vi. 1926. Duration of hydrolysis 44 hours.

p_H		Reducing sugar			
				Change	
Buffer added	At end	Experiment mg.	Control mg	mg.	%
3·0	3·0	3·5	2·0	1·5	12
3·4	3·6	5·0	2·2	2·8	22
3·8	4·2	6·0	2·0	4·0	31
4·2	4·6	8·5	2·1	6·4	50
4·6	4·9	12·0	3·8	8·2	64
5·0	5·2	14·0	4·3	9·7	76
5·4	5·3	11·5	3·5	8·0	63
5·8	5·8	17·0	4·3	12·7	100
6·2	6·4	15·0	4·3	10·7	84
6·6	6·5	15·0	4·3	10·7	84
7·0	6·9	15·0	4·6	10·4	82
7·4	7·1	13·5	4·6	8·9	70
7·8	7·3	10·5	3·8	6·7	53

Exp. 2. Leaves of *P. nigra* L. var. *pannonica*. Tashkend, 13. vi. 1926. Duration of hydrolysis 47 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·5	0·6	10	5·8	6·0	3·5	58
3·4	3·7	1·3	2·1	6·2	6·4	3·0	50
3·8	4·1	0·6	10	6·6	6·7	2·8	47
4·2	4·4	1·6	2·7	7·0	6·9	2·5	41
4·6	4·6	4·5	75	7·4	7·3	1·4	23
5·0	5·4	6·0	100	7·8	7·4	0·7	11
5·4	5·8	4·2	70				

Exp. 3. Leaves of *P. candicans*. Tashkend, 13. vi. 1926. Duration of hydrolysis 19 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·2	3·6	0·0	0	5·8	6·0	0·75	50
3·8	4·6	0·1	6	6·2	6·4	1·5	100
4·2	4·9	0·15	10	6·6	6·6	0·8	53
4·6	5·4	0·35	23	7·0	6·9	0·25	17
5·0	5·5	0·5	33	7·4	7·2	0	0
5·4	5·7	0·1	7	7·8	7·4	0	0

Exp. 4. Leaves of *S. babylonica* L. Tashkend, 13. vi. 1926. Duration of hydrolysis 45 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·0	1·0	6	5·8	5·8	13·0	76
3·4	3·6	1·6	9	6·2	6·2	15·25	90
3·8	4·0	1·8	11	6·6	6·6	17·0	100
4·2	4·3	3·8	22	7·0	7·0	13·0	76
4·6	4·6	9·0	53	7·4	7·2	12·5	73
5·0	5·0	12·5	73	7·8	7·4	9·0	53
5·4	5·4	10·75	63				

Exp. 5. Leaves of *S. oxica*. Tashkend, 13. vi. 1926. Duration of hydrolysis 46 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·2	0·1	2	5·8	5·8	3·6	61
3·4	3·7	0·5	8	6·2	6·2	4·6	78
3·8	4·2	0·6	10	6·6	6·5	5·2	89
4·2	4·6	1·0	17	7·0	6·9	5·9	100
4·6	4·9	2·7	46	7·4	7·2	3·0	51
5·0	5·0	3·5	60	7·8	7·3	2·3	39
5·4	5·4	3·0	51				

Exp. 6. Leaves of *Salix* sp.? Tashkend, 13. vi. 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·2	0·4	4	5·8	5·7	8·5	89
3·4	3·6	0·5	5	6·2	6·0	9·5	100
3·8	4·1	0·9	9	6·6	6·5	8·5	89
4·2	4·5	1·5	16	7·0	6·9	8·4	88
4·6	4·9	2·6	27	7·4	7·2	3·8	40
5·0	5·2	3·1	32	7·8	7·4	1·5	16
5·4	5·4	4·3	46				

Exp. 7. Leaves of *S. hastata*. Biological Station (Murmansk), June 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	2·6	0	0	5·8	5·5	2·5	71
3·4	2·8	0	0	6·2	6·0	2·5	71
3·8	3·2	0	0	6·6	6·5	2·5	71
4·2	3·7	0	0	7·0	6·9	3·5	100
4·6	4·6	0	0	7·4	7·3	2·5	71
5·0	4·8	0·5	14	7·8	7·6	2·0	57
5·4	5·2	1·5	43				

Exp. 7. Leaves of *Gossypium hirsutum* L. The Turkestan Cotton Breeding Station No. 182, June 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·4	0·4	10	5·8	5·8	4·1	100
3·4	3·8	0·8	20	6·2	6·2	3·1	75
3·8	4·2	0·6	15	6·6	6·6	2·4	58
4·2	4·6	0·8	20	7·0	6·9	2·2	54
4·6	4·8	2·3	56	7·4	7·2	1·9	46
5·0	5·0	3·5	85	7·8	7·4	1·4	34
5·4	5·4	2·9	71				

The optimal p_H values for the action of the salicinase of *Populus* are in general less than those for *Salix*, the greatest action of *Populus* salicinases being found at p_H 5·8–6·4 and that of *Salix* enzymes at p_H 6·0–6·9. It is of interest that the enzyme of *S. hastata* has a minimum of action at p_H 4·6–4·8 and the enzymes of middle-Asiatic species of *Salix* have a distinct action even at p_H 3·0–3·6. The values of salicin hydrolysis for this boreal species of *Salix* are generally less than for the others.

At p_H 5·3 for *P. Bachofenii*, 5·7 for *P. nigra*, 5·4 for *S. babylonica*, 5·4 for *S. oxica*, and 5·4 for *Gossypium hirsutum* it is possible to see a decrease in the hydrolysis of salicin by the enzyme. This decrease coincides probably with an isoelectric point of protoplasmic proteins, because the precipitation of proteins at their isoelectric points may carry down the enzymes and thus inactivate

them. As the investigations of Pearsall and Priestley [1923] demonstrated, the most important proteins in tissues capable of growth have isoelectric points lying between p_H 3·0 and 6·0. The isoelectric points of potato-tuber tissues and of soya-bean root-tips were found by Robbins and Scott [1925] equal to p_H 6·4 and 6·2–6·44 respectively, for the mycelium of *Giberella saubinettii* the position of the isoelectric point is at p_H 6·2, for *Fusarium lycopersici* at p_H 5·5 and for *Fusarium oxysporum* at p_H 4·9. Pearsall and Ewing [1924] give for the isoelectric point of yeasts p_H 3·1–3·3 and for *Nitella* p_H 4·6–4·7.

The relative amounts of enzyme in the leaf tissues of the plants investigated were determined by special experiments with hydrolysis of salicin at p_H values optimal for each plant species. The duration of experiment was 43 hours at 31°.

	p_H (optimal)	Reducing sugar in mg.
P. Bachofenii	5·8	11·5
P. nigra v. *pannonica*	5·4	4·0
P. candicans	6·4	6·5
S. babylonica	6·6	16·5
S. oxica	6·9	5·5
S. sp.?	6·0	8·5
S. hastata	6·9	4·0
G. hirsutum	5·8	3·5

Similar relations are also revealed by experiments on the determination of the velocity constants of salicin hydrolysis by the various leaf enzymes. These experiments give the following results (the conditions as in previous experiments).

t = time in hours.

x = reducing sugar experimentally obtained.

y = reducing sugar calculated from the formula $k = \frac{1}{t} . ln \frac{a}{a-y}$.

P. Bachofenii

t	0	12	24	36	48	End
x	0	1·75	3·75	6·5	8·5	15·7
y	0	2·3	4·2	5·9	7·3	15·7

$k = 0{\cdot}00571$

P. nigra

	0	12	24	36	48	End
	0	1·0	1·6	2·0	2·4	15·7
	0	0·8	1·5	2·2	2·9	15·7

$k = 0{\cdot}00187$

P. candicans

t	0	12	24	36	48	End
x	0	1·5	3·0	4·0	4·8	15·7
y	0	1·5	2·8	4·0	5·1	15·7

$k = 0{\cdot}00358$

S. babylonica

	0	12	24	36	48	End
	0	4·5	9·5	12·5	15·5	15·7
	0	5·6	9·2	11·6	13·0	15·7

$k = 0{\cdot}01607$

S. oxica

t	0	12	24	36	48	End
x	0	1·3	2·5	3·2	4·3	15·7
y	0	1·2	2·4	3·4	4·4	15·7

$k = 0{\cdot}00298$

S. sp.?

	0	12	24	36	48	End
	0	2·0	4·0	6·0	7·0	15·7
	0	2·2	4·0	5·6	7·0	15·7

$k = 0{\cdot}00535$

S. hastata

t	0	12	24	36	48	End
x	0	0·5	1·5	2·0	2·2	15·7
y	0	0·6	1·2	1·8	2·4	15·7

$k = 0{\cdot}00150$

The coincidence of values obtained experimentally with those calculated from a monomolecular formula is very satisfactory.

SUMMARY.

1. A definite optimal hydrogen ion concentration exists for the action of salicinases from various species of *Populus* and *Salix*.

2. The different plants contain various amounts of the enzyme.

3. The presence of salicinase in *Gossypium hirsutum* confirms the view that this enzyme is not limited to the species of the Salicaceae, which was first expressed by Krauch [1880] who found salicinase in some Cucurbitaceae.

REFERENCES.

Blagoveschenski and Sossiedov (1925). *Biochem. J.* **19**, 350.
Krauch (1880). *Landw. Vers.-Stat.* **23**, 77.
McIlvain (1921). *J. Biol. Chem.* **49**, 183.
Pearsall and Ewing (1924). *Biochem. J.* **18**, 329.
Pearsall and Priestley (1923). *New Phytologist*, **22**, 185.
Robbins and Scott (1925). *J. Agric. Res.* **31**, 385

CLXI. THE ACTION OF CARBON MONOXIDE ON CERTAIN OXIDISING ENZYMES.

By MALCOLM DIXON (1851 *Exhibition Senior Student*).

From the Biochemical Laboratory, Cambridge.

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Warburg [1926] has shown that the respiration of yeast is inhibited by carbon monoxide. Since this inhibition is prevented by light, and the combination of carbon monoxide with many complex iron compounds is known to be suppressed by light, he believes that the inhibition is due to the combination of the CO with the iron-containing "oxygen-transporting component of the respiratory ferment," which results in its inactivation. The action of carbon monoxide is thus analogous to that of cyanides.

Haldane [1927] has shown that carbon monoxide has a similar inhibitory effect on the activity of moths and cress plants, and has made it clear that this is very probably due to the poisoning of an oxidation-catalyst present in the cells.

Keilin [1927] has studied the action of CO on certain oxidising enzymes. He finds that the indophenol oxidases of both yeast and mammalian muscle are strongly inhibited by carbon monoxide and by cyanide, as is also the oxidation of catechol by the oxygenase of the potato.

In general then it would seem that carbon monoxide and cyanide act in a similar way; although the action of cyanide is usually the more marked, and fairly large amounts of carbon monoxide are necessary to produce a large inhibition (*e.g.* Warburg obtained a 60 % inhibition with 600 mm. pressure of CO).

The enzymes of the aldehyde-oxidase type (the dehydrases) have not so far been tested with carbon monoxide; and since the aerobic oxidation by certain of these systems is inhibited by cyanide, while that of others is completely unaffected, it will be interesting to determine whether carbon monoxide will show a similar distinction in behaviour towards these systems.

As convenient examples of these two classes of enzymes the aldehyde (and xanthine) oxidase of milk and the succinoxidase of muscle were chosen for investigation, since these enzymes can readily be obtained in solution free from other interfering enzymes by the methods of Dixon and Kodama [1926] and Ohlsson [1921] respectively. The oxygen uptake of the milk oxidase system has been shown by Dixon and Thurlow [1925] and by Dixon [1927] to be unaffected by cyanide; while Fleisch [1924] found that $M/1000$ cyanide

was sufficient to prevent almost completely the uptake of oxygen by the succinoxidase system, leaving its power to reduce methylene blue unimpaired.

The experiments were carried out by means of the Barcroft differential apparatus at room temperature. The carbon monoxide was prepared from formate and sulphuric acid in the usual way, and was purified by passing it through strong NaOH, then through sulphuric acid, and finally through two long U-tubes filled with adsorbent charcoal. The Barcroft apparatus was filled with the appropriate gas mixture in the following manner. After the necessary solutions had been introduced into the apparatus it was placed in position in the thermostat with the taps open, and the open ends of the tubes were connected by means of rubber pressure-tubing and a glass T-piece with a tube communicating with a graduated mercury pressure gauge. This tube was also in communication, through taps, with a vacuum pump and with reservoirs of carbon monoxide and nitrogen. The apparatus could thus be exhausted until the pressure of air remaining, as shown on the gauge, corresponded with the desired pressure of oxygen. The tap connected with the vacuum pump was then turned off, and CO or nitrogen admitted into the apparatus. The final pressure was slightly above that of the atmosphere, as the gases were stored under slight pressure, and after shutting off the supply of gas the excess pressure in the apparatus was relieved by momentarily opening a fourth tap communicating with the atmosphere. (The outlet of this tap was kept just dipping into water to act as a trap.) The taps of the Barcroft apparatus were then immediately closed, the rubber tubes detached, and after shaking for five minutes in the water-bath readings were commenced. During the whole of the filling operation, which was usually complete in under one minute, the apparatus was gently shaken in the water-bath. The connecting tubes were of fairly narrow bore, so that their volume was small in comparison with that of the Barcroft apparatus; hence dead space effects were negligible, and the actual final pressures of the various gases inside the apparatus were equal (within 1 mm.) to those given by the gauge readings. Strong NaOH solution was placed in the small receptacles provided in the apparatus to absorb any traces of CO_2 which might be given off by the solutions. The same solutions were always added to both flasks of each apparatus, except that the substance to be oxidised (hypoxanthine or succinate) was added to the right-hand flask only. In the case of aldehyde, however, it was necessary to add this to both flasks, on account of its vapour pressure, so that in this case the enzyme solution was added to the right-hand flask only. The volume of liquid in each flask was in all cases made up to 3 cc. with buffer solution.

In Figs. 1 and 2 are given graphically the results of experiments on the aerobic oxidation of aldehyde and hypoxanthine by the milk oxidase. The curves show that carbon monoxide has no inhibitory effect in either case. Several experiments were carried out both with milk and with the oxidase preparation of Dixon and Kodama [1926] and the curves given are quite typical of the results obtained.

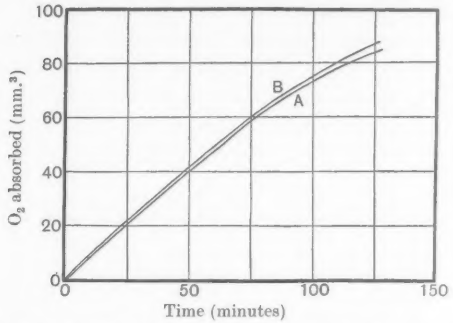

Fig. 1. Effect of CO on the Schardinger enzyme. Each apparatus contained 1 cc. fresh milk + 1 cc. phosphate buffer (p_H 7·5) + 1 cc. 0·5 % acetaldehyde. The composition of the gas in apparatus *A* was: O_2 5·3 %; N_2 94·7 %; and in apparatus *B*: O_2 5·3 %; N_2 21 %; CO 73·7 %. Temperature 16°.

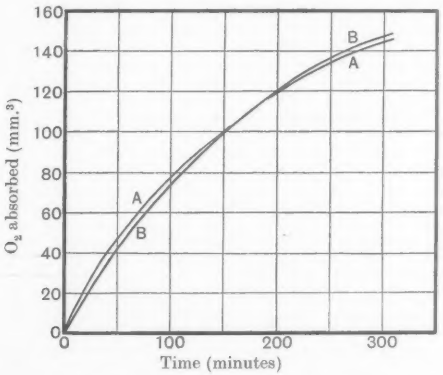

Fig. 2. Effect of CO on xanthine oxidase. Each apparatus contained 1 cc. oxidase solution + 1 cc. phosphate buffer (p_H 7·6) + 1 cc. (= 1 mg.) hypoxanthine solution. The compositions of the gas mixtures were: in *A*, O_2 5·2 %, N_2 94·8 %; in *B*, O_2 5·2 %, N_2 20·9 %, CO 73·9 %. Temperature 17°.

This negative result was to be expected, in view of the fact that cyanide has no action on these systems.

On the other hand, the fact that a small concentration of cyanide inhibits the aerobic (but not the anaerobic) oxidation of succinate by the succinoxidase would naturally be explained, on Warburg's view, by assuming the inactivation of the hypothetical oxygen-transporting iron complex present in the system; we might therefore expect that this system would show inhibition by carbon monoxide. The curves given in Fig. 3 show quite definitely however that this is not the case, the CO producing no inhibition whatever.

The enzyme solution used in this experiment was prepared from very fresh ox muscle by mincing the muscle, washing very thoroughly with distilled water, extracting with *M*/15 alkaline phosphate solution, and centrifuging

the extract, in accordance with Ohlsson's [1921] method. The experiment was repeated a number of times with different enzyme preparations, and similar results were obtained. The very slight difference between curves *A* and *B* is clearly due to a small initial temperature difference; after the first few minutes the two velocities are identical throughout. Curve *C* shows for comparison the very striking inhibition produced by the addition of *M*/1000 cyanide.

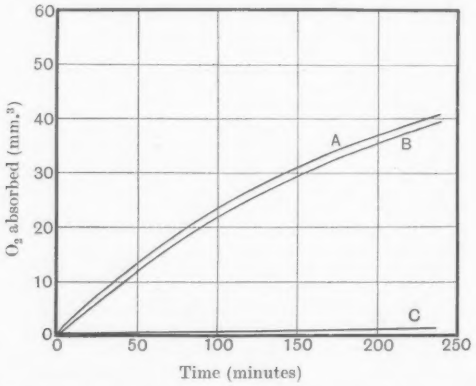

Fig. 3. Effect of CO on succinoxidase. Each apparatus contained 2 cc. succinoxidase solution (in phosphate p_H 8)+1 cc. sodium succinate solution (=2 mg. succinic acid). The compositions of the gas mixtures were: in *A*, O_2 2·6 %, N_2 97·4 %; in *B*, O_2 2·6 %, N_2 10·5 %, CO 86·9 %; in *C*, O_2 21 %, N_2 79 %. Curve *C* shows the effect of adding *M*/1000 KCN. Temperature 11°.

It is clear from these experiments that in the succinoxidase system the action of the cyanide is upon some substance which is not inactivated by CO.

These results make it clear that the inhibiting effect of carbon monoxide on the utilisation of oxygen by biological oxidation systems is (like that of cyanide) by no means universal, as Warburg seems to imply, since at least three typical biological oxidation processes are unaffected by it; and, secondly, they show that cyanide and carbon monoxide do not necessarily always inhibit the uptake of oxygen by acting in the same way or on the same substance. If it is assumed that cyanide inhibits the oxidation of succinic acid by inactivating an oxygen-transporting substance, and that carbon monoxide inhibits tissue respiration by acting in the same manner, it follows from these results that different oxygen-transporters must be concerned in the two cases.

Summary.

Carbon monoxide has no inhibitory effect on the aerobic oxidation of aldehyde or hypoxanthine by the milk oxidase, or of succinic acid by the succinoxidase of muscle. Its action thus differs from that of cyanide, which strongly inhibits the latter reaction.

I should like to express my sincere thanks to Sir F. G. Hopkins for his interest in this work, and also to Dr Keilin for suggestions and for the use of apparatus. I am also much indebted to Mr Tate for preparing the CO and for assistance with the manipulations.

REFERENCES.

Dixon (1927). *Biochem. J.* **21**, 840.
Dixon and Kodama (1926). *Biochem. J.* **20**, 1104.
Dixon and Thurlow (1925). *Biochem. J.* **19**, 672.
Fleisch (1924). *Biochem. J.* **18**, 294.
Haldane (1927). *Nature*, **119**, 352; *Biochem. J.* **21**, 1068.
Keilin (1927). *Nature*, **119**, 670.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Warburg (1926). *Biochem. Z.* **177**, 471.

CLXII. THE EQUATION OF ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN and FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute, London.

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The fact that the hexosediphosphate which is produced by the action of yeast preparations on a mixture of a hexose and a phosphate is accompanied by a monophosphate renders it necessary to reconsider the equation originally proposed by Harden and Young [1906] to represent the reaction:

$$2C_6H_{12}O_6 + 2Na_2HPO_4 = C_6H_{10}O_4(PO_4Na_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O.$$

This equation was originally established on the basis of the ratio of carbon dioxide evolved to the phosphate which went into organic combination as experimentally observed and of the composition of the hexosephosphate produced. It was further confirmed by experiments in which the ratio was determined of the sugar added in presence of excess of phosphate to the carbon dioxide produced.

It is however obvious that if some of the phosphate and some of the sugar form a compound of a different composition (a hexosemonophosphate) experiments of the kind just indicated are not sufficient to afford a rigorous proof of the equation.

It was therefore decided to make a more detailed study of the reaction and to endeavour to determine the exact proportions of the hexosemonophosphate and hexosediphosphate produced, as well as the amount of carbon dioxide evolved.

Determination of the hexosemonophosphate and hexosediphosphate produced in the reaction.

The following method has been developed from a suggestion for which we are indebted to Dr R. Robison.

The fermentations are stopped by adding enough 25 % trichloroacetic acid to raise the concentration to 3·5 %. The liquid is filtered and the amount of free and total phosphorus in the filtrate is estimated by Neumann's method. To the rest of the filtrate is added excess of barium acetate and enough hot baryta to render the liquid just pink to phenolphthalein. Enough CO_2 is then added just to remove the pink colour. The liquid is allowed to stand in the cold room for 18 hours and filtered. The free and total phosphorus are then estimated in the filtrate.

It is assumed that in this treatment the large concentration of Ba ions present decreases the solubility of the barium hexosediphosphate to such an extent that this salt is almost completely precipitated along with the free phosphate present, whilst the readily soluble barium hexosemonophosphate is left in solution.

The amount of the latter is calculated from the combined phosphorus found in the liquid. The amount of diphosphate is estimated from the total combined phosphorus less that remaining in solution after precipitation.

Attention must be drawn to two possible errors, which affect the results in opposite directions. (1) If too much baryta is added, some of the monophosphate may be precipitated in the form of a basic salt. (2) The presence of a considerable concentration of monophosphate may enhance the solubility of barium hexosediphosphate. The extent to which these occur cannot readily be gauged.

In the earlier experiments the total free and combined phosphorus was precipitated by adding 50 % alcohol in conjunction with the barium acetate and baryta. The precipitate so formed was washed with 60 % alcohol to remove sugar, and its content of free and total phosphorus and copper-reducing power determined. From these data by means of a simultaneous equation the amounts of hexosemonophosphate and hexosediphosphate were then calculated. The results were unreliable owing to the presence of barium compounds of glucose or fructose in the washed precipitate.

Attempts were also made to precipitate the hexosediphosphate alone from the fermented liquid by the addition of brucine, but the results obtained did not agree among themselves, probably owing to the slow precipitation of free phosphate by brucine.

Determination of the CO_2 produced in the reaction.

The total volume of CO_2 produced was determined in the usual manner.

Part of this is due to the conversion of phosphoric into the stronger hexosephosphoric acids, the potassium salts of which are hydrolysed to a smaller extent in solution than the phosphates and therefore take up less CO_2 in the form of bicarbonate.

To estimate this part of the gas parallel experiments are made in each of which the same amount of phosphate is added.

To one, excess of trichloroacetic acid is added immediately after the addition of phosphate and the evolved gas measured. This gives the CO_2 combined in the form of bicarbonate at the commencement of the observation.

To the second, the same quantity of trichloroacetic acid is added after all the free phosphate has been combined and the evolved gas measured. This gives the residual bicarbonate. The difference between the volumes of gas in the two cases is taken to represent gas evolved owing to the difference in properties of the hexosephosphates and phosphates.

To reduce this correction to a minimum a mixture of four volumes of

0·6 *M* KH_2PO_4 and one volume of 0·6 *M* K_2HPO_4 was used whenever possible. This mixture can be successfully employed with yeast-juice[1]. In parallel experiments with the same sample of juice the addition of 10 cc. of 0·6 *M* K_2HPO_4 or 10 cc. of the 0·6 *M* phosphate mixture produces the same evolution of CO_2 within the limits of experimental error, when all corrections have been made. The phosphate mixture was found unsuitable for use with zymin or dried yeast, probably on account of its acidity (p_H 6·4).

Another deduction remains to be made from the total volume of gas produced. It has been assumed in past experiments, that, throughout the whole period, during which the added phosphate is undergoing esterification, a steady rate of evolution of CO_2 is maintained by the constant rate of supply of free phosphate by the action of the hexosephosphatase of the yeast preparation on the hexosediphosphate present in the liquid, as shown by the equation

$$C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = C_6H_{12}O_6 + 2Na_2HPO_4.$$

This amount cannot be ascribed to the phosphorus added and represents the amount of gas which would have been evolved in the absence of added phosphate.

Some uncertainty attaches to this calculation. The possibility exists that, in the very varied range of concentrations both of phosphate and hexosephosphate which occur in an experiment, the rate of action of the hexosephosphatase may be modified. This is obviously the case when a high concentration of phosphate is employed, since the rate of fermentation is at first considerably depressed and only regains its original value after a more or less prolonged period of induction. In Exps. 66 and 68 (Table I), for example, in which 15 and 20 cc. of 0·6 *M* K_2HPO_4 were used, on the addition of the phosphate the rates fell from 4·8 to 2·4 and 1·8 cc. per 5 minutes and did not regain their original value for 50 and 100 minutes respectively.

Hence, in determining the equivalent between hexosephosphate formed and carbon dioxide evolved only such quantities of phosphate can be used as cause no preliminary depression in the rate of fermentation.

Again, at the close of the experiment, when the concentration of hexosephosphate has become much larger than its original value, the steady rate of fermentation is frequently somewhat greater than before the addition of phosphate.

The complete conversion of the quantities of phosphate which have been used in these experiments requires from 1·5 to 3 hours. The normal rate of fermentation for 25 cc. yeast-juice is about 2–3 cc. per 5 minutes; for 4 g. zymin, 4·5 to 5 cc. and the volume equivalent to the phosphate added is 67·2 cc. for 5 cc. of 0·6 *M* phosphate.

[1] The statement made by Harden [1923] that the correction is negligible "when a mixture of five molecular proportions of K_2HPO_4 with one of KH_2PO_4 is used" should read "when a mixture of five molecular proportions of KH_2PO_4 and one of K_2HPO_4 is used."

Hence an error of 0·5 cc. in the estimated "normal" rate per 5 minutes in an experiment lasting 1·5 hours involves an error of 9 cc. or 13 %.

The addition of phosphate does not produce so great a relative increase in the rate of fermentation given by zymin as it does in the case of yeast-juice. As the result of this the correction in the case of zymin (see table) is very much higher—and correspondingly less accurate—than in that of yeast-juice.

Experimental method and results.

Experiments were carried out both with yeast-juice and zymin (yeast dehydrated with acetone). 25 cc. of yeast-juice were employed in each case + 3 g. fructose or glucose + 0·3 cc. toluene; in the case of zymin 4 g. were used + 3 g. fructose or glucose + 30 cc. H_2O + 0·3 cc. toluene.

In each case three separate quantities of the mixture were incubated at 30° until the rate of evolution of CO_2 became constant. The desired amount of phosphate solution previously saturated with CO_2 at 30° was then added to two of them, which were then treated as already described.

The third was employed to obtain a determination of the hexosemonophosphate and hexosediphosphate in the fermenting mixture at the commencement of the experiment. For this purpose trichloroacetic acid was added in order to stop the fermentation and then the same amount of phosphate as was added to the other two. In the filtrate from this hexosemonophosphate and hexosediphosphate were determined. As a check the same process was sometimes carried out with the yeast-juice or zymin without the addition of phosphate.

In calculating the results the amounts of monophosphate and diphosphate found at the commencement of the experiment were subtracted from those found at the end, the differences being regarded as the amounts of the two esters formed during the experiment. The results are contained in Table I.

Table I.

			P		CO_2, cc. at N.T.P.											
Exp. no.	Material used	Sugar	Mixture used 0·6 *M* phosphate	mg. added	Total evolved	Correction for combined CO_2	Correction for hydrolysis	Corrected vol.	Duration no. of 5 min. periods	Mg. P as hexosediphosphate	Mg. P as hexosemonophosphate	CO_2/ Combined P	CO_2/ Diphosphate	CO_2/ Monophosphate	Diphosphate/ Monophosphate
46	Yeast-juice	Fructose	10 cc. acid mixture	181·6	155·4	7·6	31·5	116·3	14	133·2	53·4	0·87	2·43	3·03	1·25
50	,,	,,	,,	181·6	148·7	5·3	23·5	119·9	14	96·7	79·6	0·95	3·45	2·10	0·61
58	,,	,,	,,	181·6	148·4	7·5	29·3	111·6	16	144·2	41·1	0·83	2·14	3·75	1·75
54	Zymin 1	,,	10 cc. K_2HPO_4	179·7	215·2	21·0	90·8	103·4	17	128·8	39·8	0·85	2·23	3·62	1·62
56	,,	,,	,,	179·7	234·3	21·9	113·2	99·2	18	123·2	43·1	0·83	2·24	3·21	1·43
60	Zymin 2	,,	5 cc. K_2HPO_4	89·85	92·6	10·4	53·9	28·3	23	50·6	35·0	0·46	1·55	1·12	0·72
62	Zymin 5	,,	,,	89·15	142·2	14·4	69·7	58·1	15	83·2	8·0	0·88	1·93	10·08	5·18
64	,,	,,	10 cc. K_2HPO_4	178·3	208·9	21·0	106·7	81·3	23	134·0	38·6	0·65	1·68	2·91	1·73
66	,,	,,	15 cc. K_2HPO_4	267·4	233·1	32·2	uncertain		29	215	44·9	not determined			2·39
68	,,	,,	20 cc. K_2HPO_4	356·6	305·6	42·6	,,		44	304·2	59·6	,,			2·55
72	,,	Glucose	5 cc. K_2HPO_4	89·15	151	12·1	75·6	63·3	17	64·4	26·8	0·96	2·72	3·27	1·20
74	,,	,,	,,	89·15	146·4	11·8	74·4	60·2	17	73·0	18·2	0·91	2·28	4·57	2·00
75	,,	Fructose	,,	89·15	136·1	11·8	68·0	56·3	15	81·1	10·1	0·85	1·92	7·69	4·00
76	,,	Glucose	7·5 cc. K_2HPO_4	133·7	184·4	16·1	93·6	74·7	23	115·4	19·2	0·90	1·79	5·37	3·00
77	,,	,,	5 cc. K_2HPO_4	90·75	137·5	12·6	65·2	59·7	15	67·9	22·7	0·91	2·43	3·64	1·50

Discussion.

Ratio of CO_2 to total P esterified.

The fact that the ratio of CO_2/P is invariably below unity seems to indicate quite clearly that some esterification has taken place without evolution of CO_2 in accordance with the requirements of the original equation. As the estimation of the total amount of combined P is susceptible of a high degree of accuracy this conclusion may be regarded as well established. This is in agreement with Meyerhof's view [Meyerhof and Lohmann, 1927] that in alcoholic fermentation a monophosphoric ester is first formed without evolution of CO_2, if it be supposed that some of this still exists in the solution (either in its original supposedly active form or in a stabilised form) when the sample is taken for analysis.

Excluding the two exceptionally low results of Exps. 60 and 64, which are discussed later (p. 1221), the average ratio for the remaining eleven experiments is 0·89, the variations being from 0·83–0·96. If we assume that Harden and Young's equation correctly represents the change which results in evolution of CO_2, this indicates that an average of 11 % of the total phosphorus which has entered into combination has been esterified without evolution of carbon dioxide.

Since moreover, as will be seen later (p. 1222), there is no evidence from these experiments that diphosphate is formed without evolution of CO_2, it is probable that this direct esterification results in the formation of a monophosphate. This process then accounts for a considerable proportion, and in some cases for the whole, of the monophosphate present at the end of the experiment.

Ratio of CO_2 to hexosediphosphate.

Experiments have been made with three different preparations of yeast-juice and with three of zymin. Both glucose and fructose have been used. It will be seen that the results vary considerably, the values for the molecular ratio CO_2/hexosediphosphate extending over a comparatively wide range (1·55–3·45). How far this is due to imperfections in the analytical methods employed and how far to variations in the conditions it is difficult to decide. Duplicate analyses of the same experimental liquid give satisfactorily concordant results. On the other hand a series of three experiments (Nos. 72, 74 and 77) with the same sample of zymin at fortnightly intervals, under what were intended to be precisely similar conditions, gave decidedly different values both for the ratio CO_2/hexosediphosphate (2·72, 2·28 and 2·43) and for diphosphoric ester/monophosphoric ester (1·20, 2·0, 1·5). The barometric pressure was slightly different on these three occasions (751, 757·9 and 753·8 mm.), but whether the resulting small differences in the amount of CO_2 retained by the solution and the p_H of the liquids would affect the result is not known.

The lowest result obtained for this ratio, 1·55, was given by a sample of zymin (Exp. 60) which had been preserved for two years and fermented sugar at a much lower rate than the freshly prepared samples. The duration of the experiment was thus considerably prolonged and the corrections to be applied to the gas volume (see p. 1218) rendered correspondingly uncertain. The same is true of Exps. 64 and 76 in which an excessive amount of phosphate was added and the duration of the experiment was unduly prolonged. Excluding these three experiments, in only one experiment out of the twelve remaining did the monophosphate exceed the diphosphate. This gave the highest value obtained, 3·45, for the ratio CO_2/diphosphate. The remaining experiments, in all of which the diphosphate exceeded the monophosphate, gave ratios varying from 1·92–2·72 (nine experiments), the average being 2·38, whilst the average of the series of ten experiments is 2·26. This average agrees approximately with the value 2 which is required by Harden and Young's equation, but the individual variations are somewhat higher than would be expected, even when regard is taken of the uncertainty of the analytical methods and of the correction applied for hydrolysis to the total volume of CO_2 evolved.

The fact that this ratio, CO_2/diphosphate, is almost without exception above the value, 2, required by the equation might, however, be simply explained by assuming that the CO_2 was produced in accordance with the equation, but that some of the hexosediphosphate was subsequently partially hydrolysed, yielding a monophosphate and inorganic phosphate, the latter of which again entered into reaction.

The monophosphate actually present in the liquid analysed would then be derived from two distinct reactions, (*a*) direct esterification (see p. 1220), (*b*) partial hydrolysis of hexosediphosphate. This idea is quite in accordance with the fact that the monophosphate isolated from yeast-juice is probably a mixture [see Robison, 1922; Meyerhof and Lohmann, 1927]. On the other hand no definite evidence of the production of a monophosphate by hydrolysis of diphosphate by yeast enzymes is at present available, although this readily occurs on hydrolysis by acids [Neuberg, 1918], and Robison [1923] has obtained evidence that it probably also occurs when the diphosphate is hydrolysed by the phosphatases of cartilage and muscle. Quite recently Neuberg and Leibowitz [1927] have actually prepared the monophosphate from the diphosphate by the action of taka-diastase. Further work on this question is in progress.

It must however be remembered that if this be the origin of part of the monophosphate, a corresponding amount of inorganic phosphate must be liberated, which again enters into the reaction, since there is very little free phosphate present at the stage when the samples are taken. The carbonic acid liberated by these secondary reactions:

$$C_6H_{10}O_4(PO_4H_2)_2 + H_2O = C_6H_{11}O_5(PO_4H_2) + H_3PO_4$$

$$2C_6H_{12}O_6 + 2H_3PO_4 = C_6H_{10}O_4(PO_4H_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O$$

is comprised in that entered in the table under the heading "Correction for hydrolysis" and should, if the idea under discussion is correct, be not less than equivalent to the part of the monophosphate formed by hydrolysis. This is the case in every instance except that of Exp. 50, the exceptionally high ratio of CO_2/diphosphate and the large production of monophosphate in which remain unexplained.

Further light on the subject could probably be obtained by making a series of estimations of the relative amounts of monophosphate and diphosphate present at various stages of the fermentation, and efforts are being made to do this. Experiments of this kind as regards the total esterification have been made by Euler and Johansson [1913] and by Meyerhof and Lohmann [1927], but the distribution of phosphorus between the mono- and di-esters has not yet been ascertained.

The present experiments afford no evidence of the formation of diphosphate either directly or indirectly by esterification of monophosphate without evolution of CO_2 [cf. Meyerhof and Lohmann, 1927].

Ratio of diphosphate to monophosphate.

The ratio diphosphate/monophosphate is highly variable, the values obtained ranging from 0·61 to 5·18.

No definite relation of this value to any of the conditions of the experiment—concentration of phosphate (Exps. 62, 64, 66, 68), nature of sugar (Exps. 72, 74, 76, 77), nature of yeast preparation—can be made out. As already mentioned, in only two cases out of fifteen was the production of monophosphate greater than that of diphosphate, on many occasions (*e.g.* Exps. 62, 75, 76) it was very much less. The average value was 2·06. The factor controlling the relation of these two products has so far escaped detection.

Ratio of carbon dioxide to monophosphate.

The ratio of CO_2/monophosphate, like that of diphosphate/monophosphate is very variable—ranging irregularly from 1·12–10·08, with an average of 4·16. The variations are such as almost to preclude the possibility of the existence of a definite relation between the amounts of CO_2 evolved and monophosphate produced.

SUMMARY.

1. The reaction which occurs when a mixture of a hexose and inorganic phosphate is fermented by yeast-juice or zymin (yeast treated with acetone) has been re-examined and the amounts of carbon dioxide, hexosemonophosphate and hexosediphosphate produced have been determined.

2. The ratio CO_2/total P esterified is on the average 0·9, indicating that, if Harden and Young's equation be taken as correct, about 10 % of the phosphorus is esterified without evolution of CO_2. The product of this esterification is probably a monophosphate.

3. The ratio CO_2/diphosphate is on the average 2·38 but varies considerably in individual cases. The fact that this ratio is almost invariably somewhat greater than the value 2 required by Harden and Young's equation suggests that the diphosphate is originally produced in accordance with the equation, but that a part of it is subsequently partially hydrolysed, with formation of a monophosphate.

4. The ratios CO_2/monophosphate and monophosphate/diphosphate are highly variable and show no definite relations.

REFERENCES.

Euler and Johansson (1913). *Z. physiol. Chem.* **85**, 192.
Harden (1923). Alcoholic Fermentation (London), p. 43.
Harden and Young (1906). *Proc. Roy. Soc. Lond.* **77**, 405.
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
Neuberg (1918). *Biochem. Z.* **88**, 432.
Neuberg and Leibowitz (1927). *Biochem. Z.* **178**, 481.
Robison (1922). *Biochem. J.* **16**, 809.
—— (1923). *Biochem. J.* **17**, 286.

CLXIII. EXPERIMENTS ON BACTERIA IN RELATION TO THE MECHANISM OF ENZYME ACTION.

By JUDA HIRSCH QUASTEL
and WALTER REGINALD WOOLDRIDGE

(*Beit Memorial Research Fellow*).

From the Biochemical Laboratory, Cambridge.

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Introduction.

It may be urged that resting or non-proliferating bacteria do not form suitable material for experiments on the mechanism of enzyme action; for the reason that the reactions to which such bacteria give rise may be due, not so much to the enzymes as to the circumstance that they form organised systems capable of effects not brought about by constituent members of the systems.

In fact, it may be urged, a living, though not proliferating, cell may possess some property which is expressed by the reactions we are investigating and which would not be possessed by the cell were it disorganised or dead. That this criticism is groundless is shown by the following evidence.

1. If a suspension of *B. coli* be shaken with toluene or ether, the organism is no longer capable of reproduction on the usual nutrient media and therefore, so far as we can ascertain, is dead. But it can still give rise to a large number of activations, *e.g.* with formic, lactic, and succinic acids, at rates not markedly different from those induced by the normal or untreated organism.

2. The effect of exposing *B. coli* to some abnormal treatment (resulting usually in the death of the organism) is to produce, as exposure is prolonged, a step by step elimination of the various activating mechanisms, as though each mechanism were differently susceptible to the treatment. Now, were the activations of the cell due to the latter acting as an organised whole, we should expect that at some particular point the cell would become disorganised and its activations would cease. Each mechanism would be eliminated at the same time and there would be no step by step degradation. Since this is contrary to what actually occurs we cannot suppose that the activations of the cell depend upon the survival of the latter as an intact organised system.

We have indeed *no* evidence to support a contention that the reactions of the organism are dependent upon its survival as a living unit. It is true that when an organism is killed certain of its activations (usually those of the sugars in the case of *B. coli*) disappear, but the simple interpretation is that the agent responsible for the death of the organism is also responsible for the disappearance of the activating mechanisms—not that the disappearance occurs because the organism has been killed.

There can be no doubt, however, that the power of the cell to proliferate is dependent upon the intactness of (among other things) many of the activating mechanisms with which we have to deal. This is entirely to be anticipated, for we cannot expect a cell to survive, whose cellular or intracellular structures have been so changed, by exposure to abnormal conditions, that they can no longer perform activations (which, on our views, are simply an expression of the nature of these structures).

In a previous communication by one of us [Quastel, 1926] the theory was advanced that the dehydrogenations[1] effected by bacteria are primarily due to polarisations of substrate molecules induced by electric fields which characterise particular centres—the "active centres"—of cellular and intracellular surfaces. The effect of polarisation may be to activate the molecule, *e.g.* it may become a hydrogen donator or acceptor, but since activation necessitates the uptake of a critical amount of energy, it follows that not all molecules which are polarised will become activated. This will depend on the nature

[1] Such dehydrogenations do not refer to reductions brought about by glutathione or substances which are active *in vitro*; they refer primarily to substances which must be activated by the cell before their oxidation can occur.

and intensity of the polarising field and upon the electrical and chemical nature of the substrate molecules. The mechanism by which a substrate, when activated, becomes a hydrogen acceptor or donator is discussed in earlier papers [Quastel, 1926; Quastel and Wooldridge, 1927] and will not be discussed further here.

The view there put forward makes it possible to interpret in a consistent manner such phenomena of biological oxidations as β-oxidation, asymmetric oxidation of the double bond, the α-oxidation of propionic acid, the mode of oxidation of branched chains and so forth. It also indicates how it is possible to regard specificity of behaviour as belonging to the molecules themselves; it being unnecessary to postulate the existence of numerous specific enzymes related to oxidations. Thus one active centre may be able to effect the activation of a number of substrates, the chance of one molecule rather than another being activated depending upon its structure. But specificity of behaviour must depend not only on the chance of the molecule being activated at a particular centre but also on its power of obtaining access to that centre. If the structure of the molecule is such that, although it may become activated by the centre, it cannot reach the centre, or remain there for a sufficient period of time for activation to occur, it is clear that the molecule will not react. Specificity, then, is also a function of the accessibility of a molecule; adsorption and appropriate orientation are factors which help the access of a molecule to a centre.

The "active centre" is a part of, or a property of, cellular and intracellular surface structures; it is inseparable from surface structure, as it is a function of it. Some conception of its properties and mode of formation will be given in Section V. It is not necessary to believe that only surfaces of considerable magnitude are capable of possessing such centres. Any surface structure, however small, may possess such centres; this will depend upon the nature of the surface. This view is discussed more fully later.

If a surface structure be attacked by chemical or by physical means a change in the number and type of the active centres distributed over the surface will take place. The change will be dependent upon the extent to which the surface structure is changed. The centres which are least resistant to "shocks" (though the resistance must vary, we expect, to some extent with the type of "shock") will be the first to disappear as the surface structure is attacked. But it does not follow that an active centre must *entirely* disappear as a result of change. It may form a new centre, according to the nature of the change, with a smaller strength of field, or perhaps a field of quite different nature. As a surface structure is attacked, the general effect, we expect, will be that the least stable active centres are first eliminated; then come those relatively more stable and finally the most stable. Thus there will result a definite sequence of eliminations which will proceed step by step. The sequence will be the same, as a whole, for a large number of methods of treatment of the organism—but that it is unlikely *always* to be so is clear,

for individual variations, due to particular effects of an attacking reagent, will on certain occasions make themselves manifest. Thus a certain reagent may react vigorously with a certain constituent group of the active centres, rendering inert the substrates which are made accessible to the centres by means of this group. Yet the elimination of this group may not influence very markedly the activating power of these centres, and so substrates whose accessibility is unaltered are still activated. Such a reagent would be liable to give a different sequence of eliminations from that produced by another reagent.

That the general effect of reagents is to give a sequence of eliminations which remains as a whole the same is illustrated by experiments described in our last paper [Quastel and Wooldridge, 1927][1] where it was noted that a variety of different treatments of *B. coli* gave the same general order of eliminations. Experiments described in Section III will further illustrate this.

The importance of accessibility—*e.g.* adsorption or orientation—of a substrate as a factor in the determination of specificity will be discussed later, but it is as well to point out now that although a molecule may become activated at a centre it may not, in this activated state, undergo the reaction in question until it has passed through a particular phase of internal (electronic) structure or through a particular phase of stability. Various factors, such as orientation or adsorption, may influence the time necessary for the activated molecule to reach this phase. This interval of time is of importance, for it is possible that the molecule may become deactivated by collision before it has time to react. If the substrate molecule, through some property of its own structure or of that of the active centre, is unable to remain in contact with the centre for a sufficiently long period of time, its chance of reacting becomes small, in spite of the fact that the centre may have sufficient power to produce an activation. On the other hand, if there be present in the centre a grouping (or set of groupings) which will hold the molecule for a sufficient period of time reaction will ensue. This grouping is the "active group" of an enzyme. On our views it is simply one of the factors influencing accessibility of a substrate and hence influencing the specific behaviour of the active centre. The centre, *as a whole*, activates; one or more of its groupings is responsible for the access of the substrate. It may be possible to modify or destroy the centre, without necessarily altering the groupings responsible for access, *i.e.* the substrate may still be adsorbed without activation occurring. Or again, it may be possible to eliminate a group responsible for access without reducing the field strength to such an extent that it can no longer activate the molecule. The importance of this point lies in the fact that we should be able to differentiate between two molecules, activated at the same centre but each requiring a different group to make it accessible. By eliminating such a group one

[1] The references [Quastel, 1926] and [Quastel and Wooldridge, 1927] will, from this point, be represented by the signs [1] and [2] respectively. These papers should be consulted for full details of the experimental technique adopted in this communication.

substrate will be rendered inert whilst the other molecule, whose access is unaltered, may still be capable of activation by the centre. Thus the centre would have the appearance of being composed of two specific enzymes.

Specificity, then, is a function of the following factors.

1. Nature of the polarising field at the active centre.
2. Structure of the substrate molecule.
3. Orientation and adsorption of the substrate molecule at the centre; these depend also on the chemical structure of the centre itself.

It will be seen that a centre may easily differ from a neighbouring centre. Each has an individuality of its own—each may, indeed, be regarded as a specific enzyme but with a certain limited range of specificity. The two fundamental ways in which an active centre differs from a truly specific enzyme are:

(*a*) a range of specificity which is limited—characteristic of each centre:

(*b*) the identity of an active centre with a surface structure.

Willstätter [1927] states: "It seems that we must consider an enzyme to be composed of a specifically active group and a colloidal carrier.... The colloidal carrier seems to vary somewhat in its nature, but to be necessary for the stability of the active group." This statement can be regarded as an approximation to the view of active centres, it being emphasised once more, however, that on this view activation is due to the centre as a whole, probably to a composite of groups, and not necessarily to a specifically active group. The latter, whilst forming an essential feature of the centre, may be important primarily in securing the access of the substrate to the activating field.

Let us now consider the evidence which leads us to suppose that activations (dehydrogenations) due to bacteria are associated with surface action.

I. Evidence for surface action in the dehydrogenations produced by *B. coli*.

Whilst the evidence from work on enzymes points very clearly to the participation of surface action it is difficult to prove rigidly, by one particular experiment, that in the activations due to bacteria surface activity is fundamentally involved. It is only when the evidence as a whole is taken into account that there seems to be no escape from this conclusion, except by postulating a number of assumptions which are usually highly questionable and for which we have no evidence. By surface activity is meant activity by surfaces, either cellular or intracellular.

Quantitative measurements [1] have already indicated that the main site of reduction of methylene blue by a hydrogen donator is at the cell surface and for reasons given in that paper it was shown that *a site* of activation of hydrogen donators is also at the cell surface.

If a suspension of *B. coli* be shaken with toluene [2] a number of activations (notably those of the sugars and glutamic acid) are eliminated, whilst others (succinic acid, lactic acid, formic acid) are retained. We may try to explain

this purely on permeability considerations. The argument would be, presumably, that the effect of the toluene is to act selectively on the cell membrane, rendering it impermeable to the sugars but not to succinic acid, etc. Now this selectivity of action cannot be attributed merely to mechanical blocking for whereas glycerol is made inactive, α-glycerophosphoric acid is as active after as before treatment; again, methylene blue must still be freely permeable for the rates of action due to formic acid, etc., have not changed. We may then suppose that the selectivity of action is due to the formation of a film, at the surface, possessing selective permeabilities, but this cannot be true, for the formation of such a film would not allow methylene blue to penetrate it. Methylene blue does not diffuse, under the conditions of our experiments, from aqueous solution into toluene and we should expect a film of toluene at the cell surface to act like a lipoid layer which, as we know from the work of Loewe [1912], would adsorb but not dissolve the dyestuff, *i.e.* the latter would not penetrate into the cell.

We may suppose finally that there is a specific entrance into the cell for the sugars, glycerol and glutamic acid and another one for succinic acid, lactic acid, methylene blue, etc., and that toluene blocks the specific entrance for the sugars and not the other entrances. Now from the results of other experiments we can demonstrate differences between succinic acid and lactic acid, between glycerol and glucose, etc., so that if the interpretation of our results is simply to be that of specific attacks on pores specific for substrates we would have to assume a specific pore for almost every substrate—a conclusion which seems to be entirely out of the question.

Since the problem does not find a satisfactory solution simply in the consideration of changes in permeability, we may take the view that the toluene penetrates the cell and there affects certain specific enzymes, *i.e.* those related to the sugars, etc. But ether, benzene, chloroform, propyl alcohol, all have extremely similar effects to toluene, and since it is scarcely conceivable that such substances as these form definite chemical compounds with the enzymes, it is reasonable to suppose that they would act in the manner we would anticipate from such capillary active substances. We expect that they would form films upon the specific enzymes, rendering the latter inaccessible to methylene blue or to the substrates or to both. This clearly indicates, as we would naturally expect from other work on enzymes, that the specific enzymes for the dehydrogenations are associated with surfaces of some description. This does not apply only to the sugars, for experiments with propyl alcohol indicate that the other specific enzymes—those for succinic acid, lactic acid, etc.—are equally associated with surface action.

If the toluene does *not* enter the cell, thus affecting certain enzymes therein, we must imagine that it forms a film upon a particular patch or series of patches on the cell surface, these patches being particularly associated with the activations of the sugars, etc. If these patches were not so associated there seems to be no reason why the sugars and methylene blue should not

enter the cell at parts of the surface not affected by toluene and so react with enzymes in the cell.

Were we to ascribe the effects of treatment of *B. coli* by nitrites [2] or by high concentrations of salts [2] or by the halogens or by allyl alcohol or by sodium acrylate (Tables I and II) to changes in permeability of the cell membrane alone, we should be placed in the difficult situation of having to account for the fact that whilst a variety of donators are prevented from entering the cell, methylene blue appears to be freely permeable always. For if it were not so, formic acid could not react at its normal rate when many of the other donators have been rendered inert. Again, it seems inconceivable that such a variety of attacks should change the permeabilities of the substrates in almost exactly the same order. The simple interpretation of these facts is that the main site of reduction of methylene blue is at the cell surface, supporting the conclusion drawn from quantitative evidence.

It seems, therefore, from various lines of evidence that surface activity is intimately associated with the activations of many hydrogen donators. The evidence is based upon experiments carried out with methylene blue, and *with this dye-stuff* it appears that the outer cell surface of *B. coli* is the main site of reduction; hence this surface must constitute *one* important site of the activation of hydrogen donators. Methylene blue has been valuable in demonstrating this fact and hence in indicating clearly the importance of surface action. But it would be absurd to consider that because, with methylene blue, the outer cell surface is the main site of reduction, activations cannot be performed within the cell. The facts that mechanical disintegration of bacteria appears to have a destructive action on certain activating mechanisms [1] and that lysis of the organism by bacteriophage ultimately removes the power of the cell to reduce methylene blue [Shwartzman, 1927; Gozony and Surányi, 1925] support our conclusion that the cell surface is a site of activations, but we cannot expect that the breakdown of the cell surface will necessarily involve the destruction of all intracellular surfaces and the elimination therefore of all activating power. Our thesis has been to show that surface activity, whether it be cellular or intracellular, is fundamentally connected with the activations we are investigating and it should therefore be borne in mind that when reference is made to surface activity, the term surface includes both cellular and intracellular surfaces.

Let us now consider briefly the phenomenon of activation and the conception of active centres.

II. Activation.

It has been necessary for several reasons to consider that in homogeneous reactions a molecule cannot undergo transformation and dissociation unless it possess a certain minimum internal energy. "By internal energy is meant all the energy it possesses over and above what it would possess at very low temperatures except energy of translation, which, by the principle of relativity,

can have nothing to do with the possibility of an internal change. Any molecule containing an amount of energy equal to or greater than a critical amount may be called an activated molecule. It may not be in a position to undergo the particular transformation we have in mind for this may depend upon the orientation of the several parts of the molecule, upon the location of the energy within the molecule and upon other factors of a similar nature " [Lewis and Smith, 1925].

Two types of chemical union are recognised: the electrostatic attraction between oppositely charged ions, this is a polar linkage; and the attraction which is produced through the sharing of an electron by two atomic nuclei, this is a non-polar linkage. We suppose that for the dissolution or formation of a non-polar linkage there must be primarily a shift of the electrons from their original orbits. This necessitates the uptake of energy, the process being termed activation. A critical amount of energy of activation must be taken up by a molecule for reaction to be possible.

Ions do not require activation in ionic reactions with which we are familiar, for such reactions consist simply of rearrangements of polar linkages. There is no necessity, for instance, to postulate activation of hydrogen or hydroxyl ions.

At present two fundamental methods of activation are recognised, collision and radiation. It is unnecessary, for the purposes of this paper, to go into the processes in detail, but it is important to note the following points.

1. An activated molecule may be deactivated by collision.

2. An activated molecule may not react until a particular phase of the internal structure of the molecule has been reached [Lindemann, 1922; Thomson, 1927]. We may expect a definite time interval between time of activation and time of transformation, the interval depending on the way in which activation has been effected.

Active centres.

Much of the work on surface catalysis carried out within recent years[1] has gone to show that catalytic activity occurs at particular areas of the surface and that it varies in nature according to the nature of these areas. The areas, or active centres, constitute as a whole the active surface of the catalyst.

Adsorbed molecules at an active surface are regarded as in a distorted condition, or in a state of strain, the amount of distortion depending upon the molecular structure.

It has been shown that if a reaction catalysed by a surface occurs between two reactants both must be adsorbed and activated [see Hinshelwood, 1926].

[1] The subject is reviewed in the *Annual Reports of the Chemical Society*, **22**, 17. Owing to the existence of this report we refrain from quoting the literature concerned with surface catalysis and active centres. But fully to understand this section, the report should be read by those unacquainted with the literature.

The value of adsorption, of some description, at an active centre is obvious when it is considered that we cannot expect activation at the centre, in general, to be instantaneous. When it is considered, too, that deactivation by collision may occur it is sufficiently patent that appropriate orientation of a substrate molecule (which may lessen the time for activation to occur) and adsorption (which may give time for the activation) are factors of utmost importance in the consideration of the specificity of behaviour of active centres.

It seems to be fully established that the active areas occupy only a small fraction of the surface and that they vary in capacity both to adsorb reactants and to promote reaction.

Taylor [1925] conceives that the activity of metal surfaces is due to incomplete surface crystallisations, so that there are produced occasional groups of atoms associated with high energy and chemical unsaturation relative to the atoms in the regular crystal lattices. [In this connection see also Kistiakowsky, Flosdorf and Taylor, 1927.]

In this paper we shall adopt the view put forward previously by one of us [1] that the activations at biological surfaces occur at the active centres of these surfaces, the mechanism of activation being a polarisation of the substrate due to electric fields at the centres. The specificity of action at the centre depends upon the accessibility of the substrate to the centres, as well as upon the electrical nature of the substrate and the nature of the polarising field.

We are aware that a purely "chemical" view of activations is possible but we find such a view inadequate to interpret our results and unsatisfactory in its inability to give us a rational picture of the mechanism whereby activations are effected. Our own view can be regarded as quite definitely chemical, yet it has the considerable advantage that one is enabled to perceive more clearly the extent to which various factors can operate upon specificity of action, than by a view which calls for a new enzyme related to, and for the formation of a new unstable intermediate compound with, every new substrate which is found to be activated.

We will now enquire into some of the properties of the active centres concerned with the dehydrogenations effected by bacteria.

III. Biological active centres.

Treatment with the most diverse reagents has the effect of eliminating the activating mechanisms of *B. coli*. This leads to the conclusion that different groupings may be involved in the activation of substrates and since any one enzyme, or active centre, can be eliminated by any of the treatments, if the latter be carried out for a sufficient period of time, we must conclude that an active centre is made up of a number of groupings.

Or, if we take the attitude that the effect of various reagents is not to attack specifically certain groups in the active centres but to alter surface

structure as a whole, so that the active centres are changed or eliminated, we must still conclude that such centres are of a somewhat complex nature and not composed simply of one grouping, as, for instance, the amino- or hydroxyl-group.

It is an interesting fact that approximately the same order of eliminations occurs on submitting *B. coli* to the action of

(*a*) varying concentrations of different salts (Table II);
(*b*) bromine and iodine (Table I);
(*c*) formaldehyde (Table I);
(*d*) allyl alcohol and acrylic acid (Table I).

The action of salts on B. coli.

An examination of Table II, which gives a summary of the experimental results, will show how the same order of elimination of the substrates occurs with all the salts investigated. There are several points of interest arising out of these results.

(*a*) The reduction time for formates appears to keep almost constant in spite of large changes in the reduction times for succinate or lactate or glucose.

(*b*) The iodate ion is more effective than the chlorate ion, and the barium ion far more than the calcium, strontium or magnesium ion in accomplishing inactivations.

The fluoride ion is far more effective than the other halogen ions in attacking, particularly, the glucose-activating mechanism.

(*c*) Relatively low concentrations of salts, with the exception of barium chloride and potassium iodate, do not affect the organism. As concentration is increased the activating mechanisms are selectively eliminated, the bivalent calcium, barium and magnesium ions being more effective than the univalent potassium and sodium ions. The chlorate, bromate and iodate ions are also much more powerful in action than the chloride, bromide and iodide.

The action of bromine and iodine on B. coli.

Very small quantities of these halogens are required to effect the destruction of activating mechanisms of *B. coli*. As shown in Table I, the least resistant are those for glycerol, glutamic acid and succinic acid. Then come those for the sugars, then those for α-hydroxybutyric acid and lactic acid and finally, the most resistant, those for formic, acetic and butyric acids.

The action of formaldehyde and acetaldehyde on B. coli.

Both aldehydes have a powerful action on the activations of *B. coli*, formaldehyde at lower concentrations than acetaldehyde (Table I). The order of elimination is similar to that noted with the halogens, etc. (see Table III), but the sugar-activating mechanisms appear to be rather less resistant.

Table I.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. of the suspension of organism, and the donator, the volume being made up to 7 cc. with physiological saline solution. Reductions were carried out *in vacuo* at 45°. All substances under investigation were brought to p_H 7·4 by sodium hydroxide or hydrochloric acid. Controls with all organisms, before treatment, showed no reduction within 2 hours. The reduction times are given in minutes. The sign ∞ indicates that reduction does not occur within 3 hours. The treatment to which the organism is submitted is described at the head of each column. The organism is always well washed with saline after treatment and made up to its original bulk before use. + indicates that activation occurs, a zero sign that it does not.

	H.CHO 0·2 % 1 hr. p_H 7·4 37°	CH_3.CHO 2 % 1 hr. p_H 7·4 37°	Allyl alcohol 5 % 1 hr. p_H 7·4 37°	Acrylic acid 4 % 1 hr. p_H 7·4 37°	I_2 0·0025 % 1 hr. p_H 7·4 37°	I_2 0·005 % 1 hr. p_H 7·4 37°	Br_2 0·005 % 1 hr. p_H 7·4 37°	Br_2 0·01 % 1 hr. p_H 7·4 37°	Aniline 1 % 1 hr. p_H 7·4 37°	$KMnO_4$ 0·001 % 1 hr. p_H 7·4 37°	$KMnO_4$ 0·01 % 1 hr. p_H 7·4 37°	KCN 1% 1 hr. p_H 7·4 37°	H_2O_2 5 % of 20 vol. ½ hr. p_H 7·4 37°	Average control with organism before treatment
Succinic acid *M*/140	∞	89·5	∞	∞	122	∞	67	∞	37	92	202	49	203	13·5
Lactic acid *M*/140	16·7	32	75	52	19	180	22	140	18·7	29	15	28	50	8
Formic acid *M*/140	5	4·7	3·3	14·5	5	8	6	11	11	180	∞	∞	∞	4
Acetic acid *M*/14	8·7	6·7	4·5	29	6	11	7	16	13	180	∞	∞	∞	5·5
Butyric acid *M*/14	7·5	7·3	6·3	43	7·5	12·5	8·5	13	10	∞	–	∞	∞	7
α-Hydroxybutyric acid *M*/70	40	63·5	173	112	30	∞	30	∞	56	47	39·5	44	135	13·5
α-Glycerophosphate *M*/14	8	8·5	∞	191	6	∞	10	∞	14	150	∞	∞	∞	5·5
Glycerol *M*/7	∞	∞	∞	∞	42	∞	44	∞	∞	77	–	∞	∞	18
Glutamic acid *M*/140	∞	∞	∞	∞	62	∞	114	∞	∞	∞	∞	∞	∞	20
Glucose *M*/140	∞	36·5	∞	∞	13·5	∞	22	∞	∞	27·5	∞	15	∞	8
Fructose *M*/140	78	29	∞	∞	13	∞	20·5	∞	∞	25	–	10·5	∞	6
Mannose *M*/140	∞	44·7	∞	∞	14	∞	31	∞	∞	38	–	19·5	–	7·2
Galactose *M*/140	∞	43·3	∞	∞	13·5	∞	24	∞	∞	43	–	20·5	–	6·5
Mannitol *M*/140	∞	∞	∞	∞	20	∞	38	∞	∞	54	–	27	–	13
Sorbitol *M*/140	∞	∞	∞	∞	–	∞	41	∞	∞	51	–	27·5	–	12
Fumaric acid *M*/70	0	+	0	0	+	0	+	0	+	+	–	+	(+)	+
Na nitrate *M*/140	+	+	+	+	+	+	+	+	+	+	–	+	0	+
K chlorate *M*/140	+	+	+	+	+	+	+	+	+	+	–	+	0	+
Growth on agar from one loopful of treated organism after 18 hrs. at 37°	A few discrete colonies	Slight	None		Good	Very slight	Good	Very slight	None	Moderate	None	Prolific	Very slight	Prolific

Table II.

Reduction times of 1 cc. 1/5000 methylene blue in presence of donators (succinic acid, etc.) by *B. coli* submitted to the action of various salts at various concentrations at p_H 7·4. Organism well washed after each treatment. Conditions as in Table I.

Treatment *coli* 1	$t_{succinic}$	t_{lactic}	t_{formic}	$t_{glucose}$	Treatment *coli* 2	$t_{succinic}$	t_{lactic}	t_{formic}	$t_{glucose}$
M/5 NaCl	9′	6·25′	3·5′	4·25′	*M*/2 NaCl	16·3′	11′	3′	56′
M/5 KCl	12·5	9·25	4	7	*M*/2 KCl	20	11	3	55
M/5 KBr	12	7·5	4	5·3	*M*/2 KBr	14·5	10·3	3	11
M/5 KI	17	11·75	4·25	9	*M*/2 KI	24·5	13·3	3·3	38·5
M/5 NaF	29·5	18	3·25	24·5	*M*/2 NaF	21	16	3·5	∞
M/5 $KClO_3$	19·5	10·75	4·25	10·5	*M*/2 $KClO_3$	160	27	3	∞
M/5 $NaBrO_3$	22·5	10·5	4	14·25	*M*/2 $NaBrO_3$	150	18	3·7	∞
M/5 KIO_3	∞	35·5	4·5	∞	*M*/2 KIO_3	∞	∞	5·3	∞
M/5 $NaNO_3$	13·5	17·75	4	7·25	*M*/2 $NaNO_3$	23·5	13	3	∞
M/5 $NaNO_2$	30	18	4·5	15	*M*/2 $NaNO_2$	51	19	5·5	∞
M/5 Na_2SO_4	11·5	7	3	6	*M*/2 Na_2SO_4	15·5	13·7	3·5	∞
M/5 $(NH_4)_2SO_4$	11	7·3	3·3	5·7	*M*/2 Na citrate	15	13·7	3·7	∞
M/5 $CaCl_2$	25	11·7	4·7	105	*M*/2 $CaCl_2$	52	37	11	∞
M/5 $SrCl_2$	27	10·3	4·5	11·7	*M*/2 $SrCl_2$	67	26	5·3	43
M/5 $BaCl_2$	∞	∞	165	∞	*M*/50 $BaCl_2$	15	11	4·3	7·5
M/5 $MgCl_2$	36	16	5·5	18	*M*/2 $MgCl_2$	100	31	6·3	∞
Control	7·5	6	4	5	Control	16·5	11	3·3	6·7
M/1 NaCl	18	12·5	3·7	∞	Dist. water for 1 hour	24·5	12	3·3	9·5
M/1 KCl	29	14	4	28					
M/1 NH_4Cl	28	13	4	∞					

Table III.

Showing the general order of eliminations produced by various treatments which are described horizontally. The least stable mechanisms are eliminated first. When two or more mechanisms are eliminated together they are given the same number, and that which is next eliminated is given the corresponding number allowing for those which were previously eliminated.

	Glycerol	Glutamic acid	The sugars	Succinic acid	Fumaric acid	α-Hydroxybutyric acid	Lactic acid	α-Glycerophosphoric acid	Formic acid	Acetic acid	Sodium nitrate
Increase of temperature	1	2	3	6	6	4	5	9	9	9	8
Increase of acidity	1	1	6	3	3	5	6	9	10	10	6
Increase of alkalinity	1	2	3	4	.	5	9	5	5	5	.
Hypertonic NaCl	1	2	3	6	.	4	5	7	8	8	.
$NaNO_2$	4	3	6	1	1	4	8	7	9	9	.
Acetone	1	1	1	5	.	4	6	6	6	6	.
Benzene	1	1	1	5	.	4	6	6	6	6	.
Toluene	1	1	1	5	.	4	6	6	6	6	.
Ether	1	1	1	5	.	4	6	6	6	6	.
Phenol	1	1	1	5	.	4	6	6	6	6	.
Chloroform	1	1	1	5	.	4	9	8	6	7	.
Propyl alcohol	1	1	1	5	5	4	6	6	6	6	.
Cyclohexanol	1	1	1	1	1	1	1	8	8	8	.
Aniline	1	1	1	5	.	4	5	7	7	7	.
Methylene blue	1	1	1	5	.	1	6	7	7	7	.
Formaldehyde	1	1	1	1	1	6	7	8	8	8	.
Acetaldehyde	1	2	3	3	.	5	6	7	7	7	.
Allyl alcohol	1	1	1	1	1	7	8	1	9	9	.
Acrylic acid	2	1	3	3	3	7	8	6	9	10	.
Iodine	1	1	5	3	3	5	8	7	9	9	.
Bromine	2	1	5	3	3	6	8	6	9	9	.
KCN	3	4	6	6	6	9	10	5	1	1	.
$KMnO_4$	3	3	6	7	.	8	9	5	1	1	.
H_2O_2	1	1	1	8	8	10	11	7	4	4	4

The action of allyl alcohol and acrylic acid on B. coli.

Both these substances have very powerful effects (Table I). As usually observed, the mechanisms for lactic acid and the lower fatty acids are the most resistant to attack.

The order of elimination of activating mechanisms of B. coli.

The order of elimination noted above is similar to that already found on exposure of *B. coli* to nitrites at relatively low p_H or to different hydrogen ion concentrations [2]. See also Table III which gives a complete summary of these results on eliminations.

It would be natural to suspect that the same *order* of eliminations in such a variety of treatments is consequent upon the operation of some common factor underlying all the eliminations. This may be, as we have suggested elsewhere, a general change in the structure of the surface with the result that the most unstable centres disappear first, then those of greater stability and so on. But this view, though accounting for the same order of disappearances, cannot be entirely adequate. It does not take into account specific actions on the centres themselves, which by influencing one or more groups at the centre, may remove accessibility of certain substrates without necessarily affecting, in a marked degree, the activating powers of the centre. Were such groupings affected there would probably be a departure from the usual order of eliminations.

Such a departure from the usual order is noted particularly in the effects of exposing *B. coli* to dilute solutions of potassium permanganate or to relatively strong solutions of potassium cyanide.

The action of potassium permanganate, potassium cyanide and hydrogen peroxide on B. coli.

Here the mechanisms for formic acid and acetic acid, which are usually the most resistant to attack, are among the least resistant (see Table I). Some particular grouping, very sensitive to attack with $KMnO_4$ or KCN, would seem to be particularly associated with formic acid or acetic acid. A somewhat similar effect is produced by treating the organism with H_2O_2[1] (Table I). It may be an aldehyde or carbonyl group, but treatments with sulphites, phenylhydrazine, semicarbazide or aminoguanidine (Table IV) do not exhibit this anomalous behaviour of permanganate or cyanide.

Dilute concentrations of potassium cyanide are almost without action on *B. coli* and relatively strong solutions must be used to exhibit the eliminating action. Potassium permanganate solution, on the other hand, must be used in extremely dilute solution to show its selective effects; relatively strong solutions simply eliminate the entire activating action.

[1] The effect of H_2O_2 appears to vary somewhat with the strain of the organism, a fact which may be explained by variation in catalase content.

Table IV.

Conditions as in Table I.

Treatment	$t_{glucose}$	$t_{succinic}$	t_{lactic}	t_{formic}
Control (normal organism)	5	7·5	6	4
10 % NaCl (control), 1 hr., p_H 7·4	∞	15·5	11·5	3·7
5 % Semicarbazide, 1 hr., p_H 7·4	12	10	6	3
10 % Semicarbazide, 1 hr., p_H 7·4	∞	24	19	4·7
5 % Semicarbazide, 17 hrs, p_H 6·0	∞	∞	60	5·5
10 % NH_2OH, 1 hr., p_H 7·4	∞	∞	30	5·2
5 % NH_2OH, 17 hrs., p_H 7·4	∞	136	34	9
10 % Na_2SO_3, 1 hr., p_H 6·0	36	21	12	4·7
5 % Na_2SO_3, 17 hrs., p_H 6·0	∞	∞	31	8·7
1 % Phenylhydrazine, 17 hrs., p_H 6·0	∞	∞	50	∞
1 % Phenylhydrazine, 1 hr., p_H 7·4	>60	>60	8	>60
1 % Aniline, 1 hr., p_H 7·4	∞	63	33	6
2 % Aminoguanidine, 1 hr., p_H 7·4	32	33	19·5	4·2

Reversibility.

The action of cysteine and sodium hydrosulphite. So far as we can ascertain at present, the majority of the eliminations effected by the various treatments *B. coli* is made to undergo, are irreversible. Yet it may be that the correct experimental conditions to effect regeneration of the activating mechanisms, apparently irreversibly eliminated, have not yet been obtained and it would be unwise at present to conclude definitely that true irreversibility of action occurs. We can, however, show that some of the mechanisms attacked by potassium permanganate and particularly by potassium cyanide are capable of being regenerated. Exposure of KCN-treated *B. coli* to sodium hydrosulphite solution or cysteine (see Table V) brings about a marked recovery in the time of reduction due to formic and acetic acids. A slight recovery has been observed with the $KMnO_4$-treated organism. As noted also in our previous paper [2] the formic acid or acetic acid mechanism after treatment of *B. coli* with nitrites can be partially regenerated by subsequent treatment with sodium hydrosulphite. Such a recovery does not occur with the succinic acid or lactic acid mechanism.

Table V.

Conditions as in Table I.

Col. A. Reduction times due to *B. coli* treated with 1 % KCN soln. for 1 hr. at p_H 7·4 and 37° and finally well washed.
Col. B. Reduction times due to KCN-treated *B. coli* which has been suspended in 2 % cysteine soln. for 1 hr. at p_H 7·4 and 37° and finally well washed.
Col. C. Reduction times due to KCN-treated *B. coli* which has been suspended in 5 % sodium hydrosulphite soln. for 1 hr. and finally well washed.

Donator	A	B	C
M/28 Succinic acid	13·2	11·7	80
M/28 Lactic acid	6·2	7·2	16·5
M/23 Formic acid	60	9·5	23
M/7 Acetic acid	45	7·7	22
M/14 Glutamic acid	18·5	15·7	60
M/28 α-Hydroxybutyric acid	11·2	12	29
M/28 α-Glycerophosphoric acid	17	11·7	25·5
M/28 Glucose	5·7	6·5	17·5

The action of copper sulphate solution on B. coli *and the reversing effect of* H_2S. Exposure of *B. coli* to dilute solutions of copper sulphate (see Table VI) with

subsequent thorough washing, by centrifuging, results in the elimination of activating mechanisms of the organisms. If H_2S be passed through a suspension fo the copper-treated organism and this again well washed, the organism, which is coloured brown, is now capable of activating its substrates. The phenomenon is similar to that described by Myrbäck [1926] in the case of invertase inactivated by silver. It was thought that possibly the copper would protect the activating mechanisms from the action of toluene, aniline or phenol or methylene blue but, as the results in Table VI indicate, no such protective action occurs. It is particularly interesting in this connection that H_2S not only regenerates the activating mechanisms of *B. coli* which have been eliminated by treatment with copper, but it causes a considerable number of the treated cells which are apparently dead to become capable of proliferation. Most cells of *B. coli* are incapable of proliferation after treatment with dilute copper sulphate solution and subsequent thorough washing, but if H_2S be passed through a suspension of such treated cells, many of them now become capable of growing. We have never failed to obtain this result, though if treatment with copper has been too prolonged it is difficult to obtain regeneration of many cells by H_2S. Typical figures are shown in Table VII.

Table VI.

Reduction times in minutes with *B. coli*. ∞ = not reduced in 4 hours.

Conditions as in Table I.

			After exposing copper-treated organism to				
Donator	Normal	After treatment with $CuSO_4$ soln. (1/1000)	H_2S	toluene and finally to H_2S	1 % aniline HCl (p_H 7·4) and then to H_2S	1 % phenol (p_H 7·4) and then to H_2S	methylene blue 1/10,000 soln. and then to H_2S
Succinic acid	13	∞	164	∞	∞	∞	∞
Lactic acid	7	∞	21	20	40	134	117
Formic acid	3·5	∞	4	4	8	9·2	13
Glucose	6	∞	9	∞	∞	∞	∞

Table VII.

To 10 cc. of a suspension of *B. coli* were added 2 drops 10 % $CuSO_4$ soln. This was allowed to stand at room temperature for $\frac{1}{4}$ to $\frac{1}{2}$ hr., centrifuged and the deposit suspended in 10 cc. sterile saline. A drop of this was spread over an agar plate. The suspension was then submitted to a stream of H_2S for 30′ at room temperature and a drop again spread over agar.

Exp.	Colonies obtained from one drop of suspension after exposure to copper	Colonies obtained from one drop after passing H_2S through the suspension of copper-treated organism
1	80	800
2	59	Prolific >3000

The nature of the active centres.

It seems, from the general results on treatment of *B. coli*, that we must regard an active centre as made up of a number of groupings each of which

plays its part in determining the access of a substrate to the centre. If the surface structure as a whole is altered by a number of reagents we expect each reagent to give the same sequence of eliminations. Reagents of this class, which give the same order of eliminations, modify the fields (at the active centres) or affect the stability of the centres by a general change in the entire surface structure; reagents of the second class, which give pronounced departures from the typical order of eliminations, act by attacking particular groups at the centres. It appears to us likely that the average reagent has properties belonging to both classes.

Each centre acts as if it were a separate entity, possessing individual characteristics and, in a word, acting as if it were a separate enzyme. It differs from our usual conception of an enzyme in that it has a limited range of specificity and is inseparable from a surface structure. On such a view it is clearly as impossible for us to state the constitution of a biological active centre as it is to give the constitution of an active centre of a catalytically active alloy from only a knowledge of the constitution of this alloy. Yet it is to be remembered that a relatively small structure or colloidal aggregate may possess an active centre and it would be legitimate to state that the constitution of the structure or aggregate as a whole would be the constitution of the enzyme.

Let us now consider a hypothesis which seems to us to be the most reasonable alternative to that of active centres. The activity of a biological surface may be considered to reside in the specific actions of particular molecules which depend for their stability on a colloidal carrier or on adsorption at a surface. Thus, as soon as surface structure is affected, the molecules become affected, lose their stability and the enzymes are destroyed. Or the effect of a particular treatment may be upon certain of the specifically active molecules adsorbed on the surface. The two views—that of adsorption of specifically active molecules at surfaces which are necessary for their stability and that of active centres—are extremely closely related. It is possible, however, to obtain data which will help us to decide between these views.

IV. Some consequences of a theory of adsorbed specific enzymes.

On this view we have to imagine that there exist specifically active molecules whose stability or activity is greatly dependent upon a surface to which they are attached. That stability of adsorbed materials can be closely connected with the surface structure is sufficiently obvious from the work of Willstätter and his colleagues. The fact that the stability of an enzyme is so highly dependent on adsorption at a particular type of surface indicates that some particular association of groupings, which can easily be disturbed with resulting inactivity, is responsible for activation. The point we wish to emphasise is that activating power is unlikely to reside solely in one particular group, say $-NH_2$, which may only be primarily responsible for attaching the substrate to the molecule. Now if the specific activating power of the

enzyme molecule is dependent on the configuration of this association of groupings we must conclude that any change in the molecule which disturbs this configuration will eliminate its activity. For action to take place, the substrate must be adsorbed at the particular configuration in the molecule, and the amount of adsorption of a substrate at this configuration must be dependent on the nature of the latter. Hence, if the enzymic activity of the configuration remains intact, the power of adsorbing a substrate by the configuration must also remain intact. Any change which alters the adsorption of the substrate must necessarily change the nature of the configuration and hence the latter's enzymic activity. It should be remembered in this connection that the adsorption of a substrate at any part of the surface which is not occupied by the specifically active molecule, or even at a part of the enzyme molecule remote from the active configuration, can have little to do with the activity of the latter. The link must be between substrate and the particular configuration in the enzyme molecule.

Let us now consider that we have a system of two enzymes, **A** which is specifically related to substrate A, and **B** which is specifically related to substrate B, both A and B being hydrogen donators. To this mixture of **A** and **B** we add concentrations of A and B which are at the saturation points of their respective enzymes, *i.e.* at such concentrations that increase in concentration of either A or B makes no perceptible increase in the velocity of transformation of either of them when taken individually. Then the velocity of reduction due to a mixture of A and B will be the sum of the velocities due to A and B taken separately, unless there is preferential or reciprocal adsorption of the substrates on the enzymes. We assume that there are no complications[1] such as side reactions, etc.

Let V_A = velocity of reduction due to A
V_B = velocity of reduction due to B
V_S = velocity of reduction due to $A + B$
$V = V_A + V_B$.

Then, given no adsorption of B on **A** or A on **B**

$$V = V_S.$$

If $V_S < V$ it is possible to calculate the amount of adsorption of B on **A** or A on **B**.

1. Let there be only adsorption of A on **B**; *i.e.* none of B on **A**. Then $V_S = V_A +$ velocity due to unadsorbed portion of **B**.

Therefore velocity due to unadsorbed portion of $\mathbf{B} = V_S - V_A$.

But velocity due to total $\mathbf{B} = V_B$.

Therefore fraction of enzyme **B** which is unadsorbed

$$= \frac{V_S - V_A}{V_B},$$

since velocity is proportional to quantity of enzyme.

[1] In our experiments this is a legitimate assumption, for the amount of methylene blue used is extremely small compared with the amount of donator.

Therefore, the fraction of enzyme **B** which has been inactivated by adsorption of A

$$= \frac{V_B - (V_S - V_A)}{V_B} = \frac{V - V_S}{V_B}.$$

2. Similarly, if there be only adsorption of B on **A**, the fraction of enzyme **A** which has been inactivated by adsorption of B

$$= \frac{V - V_S}{V_A}.$$

3. If adsorption both of A on **B** and of B on **A** occurs, then

(a) fraction of enzyme **A** inactivated must be $< \frac{V - V_S}{V_A}$,

and (b) fraction of enzyme **B** inactivated must be $< \frac{V - V_S}{V_B}$.

Now if by experiment we can show that there is *no* adsorption of B on **A**, then the amount of adsorption of A on **B** must be given by the expression $\frac{V - V_S}{V_B}$. We can test this by further experiment.

According to the theory of adsorbed specifically active molecules the expression $\frac{V - V_S}{V_B}$ must remain a constant, characterising a property of the enzyme **B** (*i.e.* its power of adsorption of substrate A) and should remain unaltered whilst **B** retains activity. Thus if a surface possessing a quantity of **B** be attacked, so that part of **B** is destroyed, the remainder of **B** should still give the expression $\frac{V - V_S}{V_B}$ as representing the adsorption upon it of substrate A. Or if a portion of the enzyme molecule itself can be attacked without altering the active configuration in it the value $\frac{V - V_S}{V_B}$ should still remain constant. These conclusions depend upon the underlying assumption that a change in the structure of the active configuration of the molecule must inevitably eliminate its highly specific relationship with its substrate. To prevent any misconception of this point, which is important for our argument, it would be well to indicate that an analogy between the enzyme molecule and, let us say, a toxin is fruitless in this connection. It may be urged, for instance, that the action of a toxin may differ in a quantitative way after chemical treatment from that which occurred before such treatment and that this is an argument for supposing that we may also change the activity of an enzyme molecule purely quantitatively by treatment. But the two molecules, toxin and enzyme, must differ in one very important aspect. Both possess, it may be agreed, certain particular groups or associations of groups whose presence is necessary for the demonstration of activity, and both molecules, we may agree for the purposes of argument, are capable of being changed chemically without necessarily altering the configuration of the active groups. But in a toxin molecule, much must depend on the chemical nature of the molecule apart from the presence of the active group, in determining its access to particular structures in the body where it makes its presence demonstrable, *e.g.* permeability of a membrane for the toxin, or the solubility

of the latter. Both factors depend on the chemical nature of the molecule as a whole and clearly such factors must influence the quantitative action of the toxin.

Again, the activity of a molecule such as adrenaline is in all probability centred in one or two relatively simple groups or atoms of the molecule, and it is the chemical structure of the molecule as a whole which determines the accessibility of these groups to the susceptible structure of the body.

With an enzyme molecule, however, if a change in the chemical structure does not affect the structure of the active configuration of groups (and this would be surprising in view of the fact that the molecule is very unstable when not attached to an appropriate surface) we would have to consider that the molecule is of such a nature that a disturbance or alteration at one portion of it does not at all affect the active configuration. If this is so it is extremely difficult to perceive how such an alteration could affect the access of a substrate to the configuration itself; it could certainly be argued that if the change were such as to affect the access of substrate to the configuration it must certainly have altered matters to such an extent that the perfect "fit" between substrate and active groups has been rendered less perfect, and hence have produced a cessation of activity. Such a view is fundamental to our understanding of the cause of the high specificity of action obtained between substrate and an enzyme molecule.

We may conclude, then, that, if we can demonstrate the constancy of the expression $\frac{V-V_S}{V_B}$ however the organism or enzyme systems be treated, this will be evidence in favour of the view of adsorbed specifically active molecules. If the expression $\frac{V-V_S}{V_B}$ varies considerably this will be evidence against the view and a more adequate explanation of enzymic activity must be sought.

Experimental results.

The velocities of reduction (reciprocal of reduction times) of mixtures of various substrates are noted in Table VIII, the velocities due to substrates when alone (V_A or V_B) also being given[1]. Care was always taken that the concentration of any substrate was such that increase in concentration made no perceptible increase in velocity of reduction due to the substrate. The values $\frac{V-V_S}{V_A}$ and $\frac{V-V_S}{V_B}$ are worked out and, as explained earlier, represent the relative adsorptions of B on **A** or A on **B** respectively, assuming that reciprocal adsorptions do not occur[2].

Table IX gives the results obtained with *B. coli* which has been exposed to various treatments so that the mechanism for one or more substrates has

[1] Since it has been shown [1] that the rate of reduction of methylene blue is linear it is legitimate to take the velocity of reduction as the reciprocal of the reduction time. In our experiments a standard end-point (95 % reduction) is taken, to prevent error due to departure from linearity during the last 5 % of reduction.

[2] It is to be remembered in considering our experiments with *B. coli* that the main site of reduction is at the cell surface.

Table VIII.

Rates of reduction due to donators and mixtures of donators at their saturation concentrations. t_A = time of reduction in minutes due to A. t_S = time of reduction due to mixture of both A and B. Conditions as in Table I.

Concentrations of all donators = $M/28$, except glutamic acid $M/14$ and acetic acid $M/7$.

Exp.	A	B	$V_A=\frac{100}{t_A}$	$V_B=\frac{100}{t_B}$	$V_S=\frac{100}{t_S}$	$V=V_A+V_B$	$\frac{V-V_S}{V_A}.100$	$\frac{V-V_S}{V_B}.100$
1	Lactic acid	Succinic acid	18·2	4·3	19·6	22·5	16	67
			13·3	2·1	13·9	15·4	12	71
2	,, ,,	Formic acid	8·3	7·3	13	15·6	31	35
			6·8	9·1	13·7	15·9	32	24
3	,, ,,	α-Hydroxy-butyric acid	13·9	8·5	12·5	22·4	71	>100
			7·6	4·6	7·7	12·2	59	98
4	,, ,,	Glucose	9·1	6·2	9	15·3	69	>100
			9·3	5	9·6	14·3	50	94
5	Succinic acid	α-Hydroxy-butyric acid	3·3	11·2	11	14·5	>100	31
			4·3	16·7	16·7	21	100	26
6	,, ,,	Formic acid	2·1	14·3	13·5	16·4	>100	20
			2	13·9	13	15·9	>100	21
7	,, ,,	Glutamic acid	5·6	6·6	8	12·2	75	64
			3·3	3·1	3·9	6·4	75	80
8	,, ,,	Glucose	2·1	6·2	8·5	8·3	0	0
			2	3·3	5	5·3	15	9
9	Formic acid	,,	10·5	6·2	15·4	16·7	12	21
			12·2	5·7	15·9	17·9	16	23
10	,, ,,	α-Hydroxy-butyric acid	11·1	5·9	12·3	17	43	80
			10·9	5·3	12·5	16·2	34	70
11	,, ,,	Glutamic acid	14·3	2·6	14·9	16·9	14	77
12	Lactic acid	,, ,,	7·3	2·6	8·5	9·9	19	54
13	α-Hydroxy-butyric acid	Glucose	5·6	3	7·8	8·6	14	27
			5·9	3·7	8	9·6	27	43
14	Glutamic acid	,,	2·6	6·9	9·1	9·5	15	6
			2·6	6·2	8·2	8·8	23	9
15	,, ,,	α-Hydroxy-butyric acid	3·1	11·2	9	14·3	>100	47
			4·4	16·7	14·3	21·1	>100	40
16	Acetic acid	Formic acid	7·1	10	9·5	17·1	100	76
			8·9	14·3	13·3	23·2	100	62
17	,, ,,	Lactic acid	7·3	6·6	9·8	13·9	56	62
			6	8·3	10·9	14·3	57	41
18	,, ,,	Succinic acid	10·3	2	10·3	12·3	19	100
19	,, ,,	Glucose	10	5	12·2	15	28	56
20	,, ,,	Fructose	10·3	7·6	14·3	17·9	35	47
			9·5	6·3	13·3	15·8	26	40
21	Glucose	,,	5·7	11·1	11·1	16·8	100	51
			3·7	6·4	5·9	10·1	>100	66
22	,,	Mannose	5·7	5	5·2	10·7	97	>100
			8·2	11·5	9·5	19·7	>100	89
23	,,	Galactose	5	6	5·3	11	>100	95
			5·4	5·1	5·1	10·5	100	>100
24	,,	Mannitol	5	3·2	4·8	8·2	68	>100
			8·3	4·8	7·7	13·1	65	>100
25	,,	Sorbitol	5·7	3·4	5·9	9·1	57	93
			5·4	2	5·5	7·4	35	95
26	Fructose	Mannose	11·1	5	9·8	16·1	57	>100
			13·3	11·5	14·3	24·8	79	91
27	,,	Mannitol	9·1	4·8	8·3	13·9	62	>100
			10·3	3·3	9·8	13·6	37	>100
28	Mannitol	Sorbitol	2·5	2	2·6	4·5	76	95
			6·5	5·3	6·1	11·8	87	>100
29	Acetic acid	Glutamic acid	9·5	2·6	11·8	12·1	3	11

Table IX.

Velocities of reduction ($V = 100$/reduction time in minutes) due to donators and mixtures of donators with substrates made inactive (or partially so) by treatment of the organism. The treated organism is always washed after treatment and used under similar conditions to the normal organism. Concentrations as in Table VIII.

Exp.	Preliminary treatment of organism	*A*	*B*	V_A	V_B	V_S	$\frac{V-V_S}{V_A}.100$	$\frac{V-V_S}{V_B}.100$
1	With 0·005 % $KMnO_4$ soln.	Lactic acid	Formic acid	10	0	9·5	5	—
	,, ,, ,,	,, ,,	,, ,,	8·3	0	8·2	1	—
2	With 1 % KCN soln.	,, ,,	,, ,,	11·8	0	11·8	0	—
3	Exposure to 57° for 1 hr.	,, ,,	,, ,,	0·7	6·4	7·1	0	0
5	With 0·005 % $KMnO_4$ soln.	Glutamic acid	,, ,,	1·8	6·4	4·8	>100	53
6	With toluene	,, ,,	,, ,,	0	9·9	9·3	—	6
7	With 1 % KCN soln.	,, ,,	,, ,,	4·8	1·1	4·3	33	>100
8	,, ,,	,, ,,	Acetic acid	4·8	2·7	4·7	58	100
9	With toluene	,, ,,	,, ,,	0	8	8·5	—	0
10	With 0·005 % $KMnO_4$ soln.	Succinic acid	Lactic acid	0·5	6·4	6·6	60	5
	,, ,, ,,	,, ,,	,, ,,	1·5	10	9·8	>100	17
11	Exposure to 57° for 1 hr.	,, ,,	,, ,,	3·3	1·7	5·5	0	0
12	With 1 % KCN soln.	Glucose	Formic acid	10	0	10	0	—
13	With toluene	,,	,, ,,	0	9·9	10	—	0
14	With 0·005 % $KMnO_4$ soln.	Lactic acid	Acetic acid	6·4	0	5·1	20	—
15	Exposure to 57° for 1 hr.	,, ,,	,, ,,	0·6	3·3	4	0	0
16	With 0·005 % $KMnO_4$ soln.	,, ,,	Glutamic acid	16·7	1·8	13	33	>100
17	Exposure to 57° for 1 hr.	,, ,,	,, ,,	1·7	0	1·87	0	—
18	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	Succinic acid	Formic acid	0	6·4	6·4	—	0
19	With 0·005 % $KMnO_4$ soln.	,, ,,	,, ,,	1·5	0	1·5	0	—
20	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	,, ,,	Acetic acid	0	7·9	8	—	0
21	With 1 % KCN soln.	,, ,,	,, ,,	3·6	0	3·5	8	—
22	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	,, ,,	Lactic acid	0	5·9	4·6	—	28
23	With 0·005 % $KMnO_4$ soln.	Lactic acid	Glucose	6·4	0	5·1	20	—
24	,, ,, ,,	Glucose	α-Hydroxy-butyric acid	0	2·5	2·1	—	16

been eliminated either partially or completely whilst that for others has been retained. From these results it is possible to determine whether the expression $\frac{V-V_S}{V_A}$ or $\frac{V-V_S}{V_B}$ remains constant or not.

For instance, take the case of lactic acid and formic acid.

From Exp. 2, Table VIII,

$$\frac{V-V_S}{V_A} = 31\ \%,$$

i.e. assuming no adsorption of lactate on the formic acid enzyme, adsorption of formate effectively eliminates 31 % of the lactic acid enzyme;

or $$\frac{V-V_S}{V_B} = 30\ \%\ \text{(average)},$$

i.e. assuming no adsorption of formate on the lactic acid enzyme, adsorption of lactate effectively eliminates 30 % of the formic acid enzyme.

Now from Exps. 1 and 2, Table IX, we find that when the organism is so treated that formate is no longer activated,

$$\frac{V-V_S}{V_A} = 2\ \%\ \text{(average)},$$

i.e. there is very little adsorption of formate on the lactic acid enzyme.

Therefore $\frac{V-V_S}{V_B}$ should be about 30 %.

But from Exp. 3, Table IX, we find that when the organism is so treated the lactic acid enzyme is almost completely eliminated,

$$\frac{V-V_S}{V_B} = 0.$$

This result is contradictory to the hypothesis of adsorbed specifically active molecules.

Similarly with the cases of
formic acid and glucose (Exp. 9, Table VIII; Exps. 12, 13, Table IX);
lactic acid and succinic acid (Exp. 1, Table VIII; Exps. 10, 11, Table IX);
lactic acid and acetic acid (Exp. 17, Table VIII; Exps. 14, 15, Table IX);
succinic acid and formic acid (Exp. 6, Table VIII; Exps. 18, 19, Table IX);
succinic acid and acetic acid (Exp. 18, Table VIII; Exps. 20, 21, Table IX).

All these results clearly are in opposition to a view which states that the enzymes involved are of highly specific nature and that the fact that substrates do not act quite independently of one another in presence of these substrates is due to adsorptions of inert substrates at the enzymes[1].

But the results are in harmony with the view that active centres on the surfaces are the responsible agents for activation.

Since an active centre has a limited range of specificity it follows that after treatment of an organism, when an alteration of the centre or a rearrangement of its constituent groups has occurred, two effects will be manifest: (1) the configuration will have changed and this will result in some cases in

[1] It should be mentioned, in parenthesis, that if substrates are not used at their saturation concentrations, they appear to act independently of one another. It is highly important, therefore to perform preliminary experiments to ascertain the saturation concentrations.

the entire elimination of accessibility of substrates to the centre; (2) the nature of the activating field will have altered. This means that, after treatment, a centre may still retain activity for a particular substrate but yet the accessibility of other substrates to it may have entirely changed. The expression $\frac{V - V_S}{V_B}$ need *not* be constant, and on our views is unlikely to remain constant.

It is an interesting fact that the experiments of Table IX indicate that in many instances selective elimination of the activating mechanisms, or selective poisoning, reduces adsorption of the inactivated substrate at a centre, where it was previously adsorbed, to a value which is too small to be measured by our experimental method. This would certainly be expected if particular groups or group configurations were associated with the adsorption or orientation of certain substrates, and that these groups or configurations were selectively attacked by the various treatments the organism undergoes.

Table X.

Reduction times of 1 cc. 1/5000 methylene blue soln. due to donators (A) at their saturation concentrations in presence of other substances (B).
Conditions as in Table I. Normal *B. coli*.

A	B	t_A alone	t mixture of A and B
Succinic acid $M/28$	Caprylic acid $M/14$	15	33
,, ,,	Nonylic acid $M/14$	15	∞
,, ,,	Adipic acid $M/84$	21·2	29·4
Formic acid $M/28$	Propionic acid $M/14$	9·3	9·7
,, ,,	Caprylic acid $M/14$	9·3	9·0
,, ,,	Nonylic acid $M/14$	9·3	14
Acetic acid $M/7$	Propionic acid $M/14$	10·0	10·5
,, ,,	Caprylic acid $M/14$	10·0	9·5
,, ,,	Nonylic acid $M/14$	10·0	64
Glucose $M/28$	Caprylic acid $M/14$	6·7	∞

It can be shown that retardations of velocities of reduction are effected by substrates which are *not* perceptibly activated, *e.g.* caprylic and nonylic acids (see Table X) so that it is sure that adsorption can occur at active centres of substances which are not activated by them. Again some inactivated donators are adsorbed at active centres as indicated in Exps. 22–24, Table IX. These facts considered in conjunction with the fact that an approximately similar order of eliminations occurs with many diverse treatments (Table III) point, as we have observed elsewhere, to some underlying factor, concerned with the energetics of the field, or with the stability of the centres at the surface, playing a significant part in the eliminations.

V. General considerations.

Formation of active centres at biological surfaces.

There is, naturally, great vagueness at present as to the mechanism of formation of enzymes and their relationship to the remaining constituents of the cells, but it seems to be fairly widely held that the cell is able to elaborate at least two distinct classes of molecules, the very highly specialised molecules

which exhibit enzymic behaviour, and the enzymically inert substances which together make up the protoplasmic and histological structures of the cell. This view, which calls for a sharp line of demarcation between the architectural units of the cell, proteins, nucleotides, etc., and the specific enzymes, which are not only being synthesised themselves in the cell but which are regulating the course of metabolism and the growth of the cell itself, seems to be greatly strengthened by the fact that a large number of enzymes can be secreted by the cell. This gives the impression that enzymes are simply products of the cell in much the same way as, let us say, adrenaline.

When it is considered (*a*) that the number of highly specialised enzyme molecules, which the cell is presumed to contain, must be very large indeed and that they must vary considerably in their type and constitution, (*b*) that the evidence, presented in this paper, concerning the dehydrogenations effected by bacteria is contrary to the supposition that activity is due to the presence of many highly specific enzyme molecules, (*c*) that it is extremely difficult to understand how a cell is able to cope with material to which hitherto it has not been accustomed, if its content of specific enzymes, though large, is yet limited, it will be granted that the view stated above stands in need at any rate of some emendation.

The hypothesis we put forward, that enzymic activity may be regarded as the property of the active centres of cellular and intracellular structures (and this includes the smaller structures capable of extraction from or secretion by the cell) leads to a considerable simplification of the above view. Precisely what enzymic behaviour a particular structure or colloidal aggregate in the cell may possess depends on the nature of the active centres which form a part of the structures or of the colloidal aggregates. Enzymes, on this view, are themselves part of and cannot be dissociated from the architectural units of the cell. This does not imply, of course, that only the histological structures are involved; the smaller colloidal aggregates are just as much part of the architecture of the organism. The conditions existing in the cell are such as to bring about just that arrangement or juxtaposition of molecules which makes for the formation of active centres on the normal material of the cell. Thus we may imagine that the protein, nucleotides, etc., are not only so arranged as to form the various substances of the cell but that the arrangement is such that active centres are formed on these particular substances. Enzymes, therefore, and cellular structures are inseparably connected. It is necessary to consider, now, how the active centres are formed.

Let us consider the formation of molecular structures at an interface. This may be accomplished in either of two ways.

1. As new molecules are produced they will orientate themselves in a definite way determined by the forces at an interface and will tend to arrange themselves into a crystal lattice pattern. Now if a number of dissimilar molecules are engaged in forming a surface structure we expect that there will be produced points of weakness or of loose fitting. It is at these points that

stray fields occur, the fields being balanced by surface forces and constituting the active centres of the surface structure.

2. We may conceive of the production of a colloidal aggregate which is represented in a highly diagrammatic manner in Fig. 1. Here various molecules, represented by rectangles, are joined to each other by arrows which represent the connecting links between molecule and molecule. The link may be brought about by electron sharing, or the positions of the molecules relative to each other may be determined by orientating forces in the phases surrounding the molecules. The molecules will tend to take up positions so that the potential energy of the system is at a minimum.

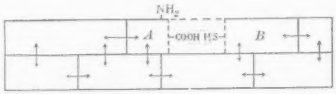

Fig. 1. Illustrating the colloidal aggregate (sectional).

Now let us consider two particular constituents, A and B, of the aggregate. It may happen that were the molecule A free from the restraint of the neighbouring molecules of the aggregate, it would form a structure represented by the skeleton scheme (Fig. 2). Here the external field of the molecule would be at its minimum; "affinities" between atom and atom in it are saturated as far as possible. But when forming part of a structure the action of the other molecules might tend to strain the configuration. This is illustrated in Fig. 3 where we suppose that two constituent groups M and M' of the molecule A are held or attracted by neighbouring molecules in the aggregate, so that a strain occurs, pulling two other constituents X and Y considerably apart. Such a strain must cause the production of an external field between X and Y, much greater than that which normally occurs. To take a concrete example,

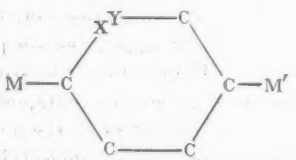

Fig. 2. Illustrating the normal character of molecule A with external field between X and Y at a minimum.

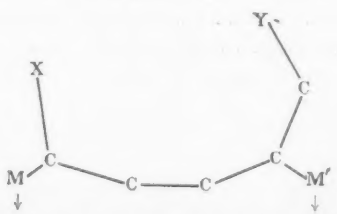

Fig. 3. Illustrating shift in relative positions of X and Y (with resultant production of increased external field) due to action of surface forces at M and M'.

in Fig. 1, X and Y are represented by the amino- and carboxyl groups. These would normally have considerable affinity for each other and tend to approach to a critical distance. The strain imposed by the neighbouring molecules of the surface structure and surrounding phase causes a widening of the distance between the two groups and it is here that we may expect the local production of an external electric field. The carboxyl group at A may link with, say, an —SH group at B, to form continuity of the surface, but this link, being perhaps much weaker than that normally between carboxyl and amino-group, will not necessarily reduce the field at A to any considerable extent.

The active centre we would imagine in the molecule $-A-\mathrm{CO}-\mathrm{S}-B-$ with $\mathrm{NH_2}$ attached to the CO group to be made up at least of the groups $-\mathrm{NH_2}$, —CO— and —S— and each of these groups will play its part in rendering a substrate accessible to the centre. The field due to these groupings may not be homogeneous and hence the orientation of a substrate at the centres, so that activation may occur, becomes a highly important consideration.

We have given this illustration of a possible formation of an active centre in order to make clear the difference between the centre and the usual conception of an enzyme. The actual composition of an active centre will in all probability be much more complex than in the illustration given. We may regard the entire aggregate as the enzyme, or the particular centre (at the link) as the enzyme. Each view is equally legitimate. But the residue of the aggregate, distinct from the region occupied by the centre, may be the seat of other active centres, so that the aggregate as a whole may have a much wider range of specificity than were the residue inert in this respect. Such an aggregate would be a relatively large colloidal particle and it would certainly be difficult to regard it as a *specific* enzyme. On the other hand, the residue may be of comparatively small dimensions and contain no other active centres. The specificity of action would be determined by the single centre and the range of specificity may be so small as to make the particle a highly specific enzyme. In this manner we regard the "soluble" enzymes[1] as related to the "insoluble." The distinction between them is simply one of degree.

Stability of active centres.

In the first place there can be little doubt that the centre, represented, let us say, by the illustration (Fig. 1), will have a limited range of stability. Its stability simply rests upon the operation of forces at the interface and we expect any factor which disturbs these forces to result in a disturbance of the links between A and B and other molecules of the aggregate with the consequent elimination of the centre. Any agent which has the effect of altering surface structure will have a "poisoning" action on the centres, though the

[1] It is to be remembered that only heterogeneous catalytic systems are being discussed.

poisoning may be selective according to the nature of the centres. We expect the most unstable centres to disappear first, the more stable next and so on. The second type of poisoning action will be on the groupings constituting the centre itself, *i.e.* one of these, say —CHO, may link up with the poison eliminating the access of some particular substrate to the centre. But the field, though changed, may still be capable of activating other substrates accessible to the centre. We expect that such a poisoning action may be reversible.

The actual size of the aggregate can give us little information on the stability of the centres. It may be small enough to be classed as a "soluble" enzyme and yet have relatively considerable stability; it may be large enough to be classed as "insoluble" and yet be extremely unstable. We can be sure, however, that any means which will strengthen the forces at the interface in preventing fission of *A—B* will increase the stability of the centre. There appear to be at least the following ways of accomplishing this.

(*a*) By adsorption of the aggregate at an *appropriate* surface, which may tend to keep the molecules in position and prevent rupture by collision.

(*b*) By the adsorption or combination at the centre, itself, of some substrate, which is reversible in action and which may protect the centre. For instance, adsorption of sucrose by invertase has a protective action on the enzyme.

(*c*) By reducing the chance of rupture of *A—B* by impact, *e.g.* by increasing the viscosity of the medium in which the aggregate is present.

Co-enzymes.

It is clear that any substance may be classed as a co-enzyme which (*a*) will tend to make a substrate accessible to the centre by combination with, or by facilitating a combination with, the substrate, the resulting combination possessing a lower energy of activation or increased adsorption or a more appropriate orientation at the centre, or which, (*b*) by some action at the centre itself, increases the activating power of the latter or the accessibility of the substrate. Such co-enzymes may not only make activity demonstrable where otherwise it could not be perceived but may increase the range of specificity of the active centre or enzyme.

Anti-enzymes.

We cannot expect that a molecule of the type *A—B* (Fig. 1), whose enzymic activity is dependent upon surface forces and the nature of the union between *A* and *B*, *must* give rise to an antibody which will necessarily eliminate the activity of the enzyme. We may expect the production of antibodies which may act as precipitins, neutralising the charge of the aggregate as a whole, but such combination does not in the least indicate that the active centre will be destroyed.

SUMMARY.

1. An attempt based upon the conception of active centres is made to interpret the mechanism of enzyme action. This conception, which is a development of views put forward earlier [Quastel, 1926] on the mechanism of biological dehydrogenations, is discussed in relation to the specificity of enzyme action and to the behaviour of enzymes when exposed to various changes in chemical and physical environment.

2. Results are given which show that a hypothesis which claims that the activity of surfaces is due to the adsorption thereon of specifically active molecules is inadequate.

3. A suggestion is made as to the mode of formation of active centres (or enzymes) in the cell. It is shown upon what factors their stability depends and in what ways their activity is influenced.

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REFERENCES.

Gozony and Surányi (1925). *Zentr. Bakt. Par. Abt. I Orig.* **95**, 353.
Hinshelwood (1926). Kinetics of chemical change (Oxf. Univ. Press), 161.
Kistiakowski, Flosdorf and Taylor (1927). *J. Amer. Chem. Soc.* **49**, 2200.
Lewis and Smith (1925). *J. Amer. Chem. Soc.* **47**, 1508.
Lindemann (1922). *Trans. Faraday Soc.* **17**, 598.
Loewe (1912). *Biochem. Z.* **42**, 190.
Myrbäck (1926). *Z. physiol. Chem.* **158**, 160.
Quastel (1926). *Biochem. J.* **20**, 166.
Quastel and Wooldridge (1927). *Biochem. J.* **21**, 148.
Shwartzman (1927). *Zentr. Bakt. Par. Abt. I Orig.* **101**, 62.
Taylor (1925). *Proc. Roy. Soc. Lond.* A, **108**, 105.
Thomson (1927). *Phil. Mag.* (VII), **3**, 241.
Willstätter (1927). *J. Chem. Soc.* 1374.

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